nature portfolio

Peer Review File

CYpHER: Catalytic extracellular targeted protein degradation with high potency and durable effect

Corresponding Author: Dr Natalie Nairn

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)
In this manuscript, Crook et al. reported a new technology for targeted protein degradation. By utilizing pH-dependent release and the rapid-cycling transferrin receptor, this new technology, called CYpHER, is designed to deliver drugs specifically to the target while recycling the drug. The authors applied this technology to target EGFR in lung cancers. While this study is very interesting and potentially impactful, there are multiple major concerns about the experiments in treating NSCLC cells and xenografts.

(1) A549 and H358 are KRAS mutant NSCLCs that do not depend on EGFR. What is the rationale for investigating those cells? Why EGFR degradation in those cells leads to cell death? Are the drugs (e.g., CT-1212-1) EGFR specific or have off-targets? Why not focus more on cancer cells with EGFR-activating mutation or amplification?
(2) As the authors already noted, skin toxicity is indeed a concern when targeting EGFR. The in vitro analyses comparing the effects of drugs on H1975 and keratinocytes are not convincing to reduce these concerns. Authors should test this in mice. For example, are the doses needed for tumor inhibition toxic to the skin?
(3) Figure 8. The in vivo efficacy of CT-1212-1 is not convincing. Authors should compare the in vivo efficacy of the new drugs to Osimertinib, a drug used in the clinic for EGFR-mutant NSCLC (e.g., H1975). Long-term experiments, including tumor growth curves and mouse survival, are needed to show the efficacy of CT-1212-1 and/or CT-1222-1 in vivo. In addition to Ki67 and EGFR, authors should also analyze cell death markers and pEGFR/pERK. The toxicity of the drugs should also be evaluated. Finally, authors should include additional mouse models to confirm the major conclusion (e.g., tumor growth inhibition).

Reviewer #2

(Remarks to the Author)
In this work, Nairn and colleagues developed a CYpHER technology that allows for durable lysosome-mediated degradation of target proteins in a catalytic manner. Compared with molecular glues and PROTACs that drive protein degradation catalytically, a key limitation for lysosomal pathway-dependent degraders is their perish together with the protein target, rendering them less durable in efficacy. Nairn et al. addressed this challenge by imparting the recycling behavior of Tf-TfR complex between the cell surface and endosomes back and forth to CypHERs. The concept of this work is interesting and such design may represent as an iteration for TPD drugs. While I acknowledge the novelty and comprehensive experimental design of this work, the unsatisfying quality of some data, the lack of statistical analysis throughout the manuscript, and concerns about biological reproducibility preclude me from recommending acceptation in its current form. Besides, more lines of evidence pertaining to the recycling characteristic of CYpHERs as well as investigation on their valency effect on protein degradation should be provided.

1.The authors described that CYpHER molecules were constructed on the basis of cystine-dense peptide (CDP) miniproteins. Explanations are needed to elucidate why CDPs were chosen as the building block? What properties do CDPs have?
2.I have to say most, if not all, statistical data presented in this manuscript lack significance analysis, which dampens readers' confidence on the validity of relevant results. In some cases (Fig. 1F), fold changes in each group should be marked for better comparison.
3.In Fig. 2B and 3D, is there any evidence or biological basis to illustrate that the GFP tag is localized outside

endo/lysosomal cavity? If so, the GFP should not be degraded to show fluorescence decay.
4.The quality and resolution of confocal fluorescence images shown in Fig. 2C-E are poor (some images are blurry; all images lack scale bar). Please refine these data. In addition, illuminating the locations of lysosomes through a costaining assay would be more informative.
5.I strongly recommend the authors to provide western blot results along with their flow cytometric data shown in Fig.2G,I,K, which is necessary to directly prove the degradation of total PDL1 proteins by CyPHERs.
6.What conclusion could be drawn from the comparison between CT-4212-1 (flexible linker) and CT-4121-3 (rigid linker)? There seems no instructive information pertaining to the design of linkers in CYpHER molecules.
7.What is the purpose to zoom in the HPLC spectrum in Fig. 3c?
8.The western blot result in Fig. 3F should be quantified to mark degradation efficiency.
9.All microscopy images should be marked with scale bars.
10.It looks abnormal in Figure 3J that lysosomes, as illuminated by the CellLight Probe, are so sparse, with no more than three “dots” in each cell. The authors should explain this.
11. I appreciate the well design of the catalytic uptake assay that demonstrated the catalytic mechanism of action for CYpHERs. However, more characterizations are needed to reveal this recycle process. For example, what is the decay kinetics for cell surface-retained CYpHER molecule? How long the degradation effect can be maintained after the withdrawal of CYpHERs?
12.In Fig. 4G, how to understand the rebound of EGFR expression in some cell lines after withdrawal? How to exclude the intrinsic difference across cell lines in their ability to recover EGFR expression to baseline? A control degrader without pH sensitivity will be helpful to address this issue.
13.It seems that monovalent degraders, e.g, 1211-1 and 1112-1, showed lower degradation efficiencies than divalent 1212-1 after withdrawal (Fig. 5B). Quantitative results of surface and total EGFR levels corresponding to Fig.5B should be provided. If so, the valency effect of CYpHERs on degradation efficiency should be carefully discussed.
14.Why the authors compartmentalized western blot results in Fig. 7B and C? Were those stripes in different groups obtained from the same gel and imaged under identical exposure settings? Uncropped original gel images should be provided for check. In addition, pEGFR levels in different groups after withdrawal should also be evaluated.
15.The authors designed five CYpHER formulations in the animal experiments, as shown in Figure 8A, but some key control groups, such as CT-1232-1/1211-1, are missing in the final efficacy evaluations (Figure 8E,G). In addition, why the degrader with rigid linker (5212-3) showed comparable degradation effect with its flexible linker counterpart, but has no effect on Ki67 inhibition?

Reviewer #3

(Remarks to the Author)
The authors described a very interesting system for a targeted protein degradation strategy which can be applied for targeting to tumors, including the ones localized in CNS.
The work is original and manuscript is well described and discussed. However, some points must be addressed especially in consideration of the applicability of the system to the clinic.

Major concerns:

The choice of the transferrin receptor as a capture molecule is rational because it is advantageous from the point of view of degradation of the target molecule, however it entails a series of implications that should be verified and discussed.

Neither in this work, nor in the previous one in which the specificity of the binding with TfR is demonstrated, there are data on the effect of this system on iron metabolism.
The authors demonstrate the biological effect of the treatment on the target molecule but do not demonstrate whether there is a side effect on iron metabolism. What effect does it have on erythropoiesis? If the system is selected to last over time, could this induce iron deficiency?

Transferrin Receptor 2 is homologous molecule of TfR1, it has a role in modulate iron homeostasis and loss of its function results in excess circulating iron and developed of a disorder called hemochromatosis. Has it been tested whether CYPHER also binds TfR2?

The lack of statistical analysis does not seem to make possible an accurate comparison of the data.

Minor point:

In the abstract it seems that the degradation system is valid in general while it refers to membrane proteins, it should be specified better.

*Continued review report:

The manuscript is a very interesting piece of work that demonstrates an innovative and potentially very useful therapeutic approach.

Major

Although the data presented are quite extensive and convincing, I believe it is essential to employ more statistical tests. Specifically:

- Fig. 3: Statistical analysis of H and I. Especially to see if the levels of Surface EGFR are statistically different in different intervals or if the value remains constant.

- Fig. 4: Statistical analysis of B, C, E, F, G, and H. A more comprehensive statistical model, considering also the different cell lines, could provide valuable insights into a potential mechanism linking CYpHER activity to the number of TfR and/or EGFR receptors.

- Fig. 7: D-I. Due to large variance among initial points, when the concentration is practically 0, I deem it essential to conduct more detailed statistical analyses. The comparison with "standard" therapies is arguably one of the most important data points. Additionally, it is crucial to understand whether the decrease in cell levels is due to the internalization and degradation of EGFR or, for example, due to difficulties in the iron supply of these cells resulting from the decrease in TfR (as indicated in fig. S4).

Minor

- Fig. 6: Statistical analysis of C, D, and E.

- Conduct statistical analyses of the graphs in fig. 2 (F-K). Additionally, it would be useful to perform quantitative analyses of images C-E.

- Figure S4 shows fluctuations in TfR levels, which are labeled as "Mild" for some cells, which indeed have fewer TfR: however, it is not clear how it is possible that in some conditions (A549:1hr and H1975:3day) the number of TfR receptors is even higher than in untreated ones. My concern is that, rather than being caused by the process described by [47], it may be a variation that does not exist (a simple statistically insignificant variation). I would encourage the authors to conduct more in-depth statistical analyses and to better comment on the result.

Reviewer #4

(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.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have conducted additional experiments and offered well-reasoned explanations in response to my comments.

Reviewer #2

(Remarks to the Author)
After looking through the revised manuscript, I appreciate that the authors have appropriately addressed my major concerns by providing sufficient experimental evidence or reasonable explanations. As this work indeed reported a significant technical advancement in TPD-related fields, the novelty of which has been concordantly acknowledged by all the reviewers, I am pleased to recommend its publication in Nature Communications.

Reviewer #3

(Remarks to the Author)
In the revised manuscript the authours have answered to our concerns.

Reviewer #4

(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.

Crook et al., "CYpHER: Catalytic extracellular targeted protein degradation with high potency and durable effect"
Nature Communications submission
Responses to Reviewer Comments
05-Aug-2024

REVIEWER COMMENTS

Author responses in italics.

Reviewer #1 (Remarks to the Author):

In this manuscript, Crook et al. reported a new technology for targeted protein degradation. By utilizing pH-dependent release and the rapid-cycling transferrin receptor, this new technology, called CYpHER, is designed to deliver drugs specifically to the target while recycling the drug. The authors applied this technology to target EGFR in lung cancers. While this study is very interesting and potentially impactful, there are multiple major concerns about the experiments in treating NSCLC cells and xenografts.

(1) A549 and H358 are KRAS mutant NSCLCs that do not depend on EGFR. What is the rationale for investigating those cells? Why EGFR degradation in those cells leads to cell death? Are the drugs (e.g., CT-1212-1) EGFR specific or have off-targets? Why not focus more on cancer cells with EGFR-activating mutation or amplification?

We investigated A549 and H358 cells (in addition to KRAS wt H1975 and H1650) knowing that the effects of an EGFR inhibitor could rationally be expected to be modest on downstream growth pathways, but as they are cancer models that express EGFR, they can be instructive in the dynamics of EGFR trafficking and depletion in response to CYpHER.

The question about why EGFR CYpHER suppresses growth in KRAS mutant lines was a curiosity to us as well. Since initial submission, we've conducted additional characterization of the cell growth suppression effects. We have determined that cells in the in vitro environment are especially sensitive to iron deprivation and that EGF can stimulate phenotypic changes in a KRAS mutant line. The growth inhibition phenotype can be suppressed by iron supplementation in the media (via 2 μM ferric ammonium citrate [FAC]). We have noted this and included the relevant data in the revision, and re-wrote the growth inhibition section to account for this. We also noted that CYpHER suppressed two phenotypes that occur in A549 cells when 1 ng/mL EGF is present, an amount published as relevant to the tumor microenvironment, in addition to FAC. A549 cells adopt a more migratory, less colony-based growth phenotype when EGF is present, and they also show a modestly increased growth rate. In the presence of FAC, both of these EGF-dependent phenotypes were suppressed by EGFR-targeting CYpHER but not by a control molecule that only binds TfR. This demonstrates an effect of EGF:EGFR on growth and migration of A549 cells that is not provided by the KRAS G12S mutation alone, and that this effect can be suppressed by EGFR CYpHER. Finally, while lung cancer is not typically treated with EGFR-targeting biologics, it is standard of care in KRAS wild type colon cancer, so we tested the KRAS wild type colon cancer cell line SW48. While SW48 cells showed reduced growth in vitro in response to CYpHER (but not cetuximab), the effect was only partially suppressed by FAC (as opposed to the complete suppression seen in KRAS mutant A549). This provides further support for CYpHER-mediated EGFR inhibition altering cell behavior in vitro beyond TfR disruption.

We will also note that this acute sensitivity to iron deprivation is not obviously apparent in vivo, as only some CYpHER designs suppressed growth in vivo (Fig. 8G). Additionally, there are no obvious effects of CYpHER on reticulocyte levels upon chronic dosing; reticulocyte levels are known to be sensitive to some TfR-engaging drug designs. This data is in the revision. We therefore believe the sensitivity to iron deprivation to be particularly acute in cancer cells grown in vitro with standard serum-based media, though more studies would be necessary to better characterize this phenomenon.

(2) As the authors already noted, skin toxicity is indeed a concern when targeting EGFR. The in vitro analyses comparing the effects of drugs on H1975 and keratinocytes are not convincing to reduce these concerns.

Authors should test this in mice. For example, are the doses needed for tumor inhibition toxic to the skin?

This question that has been on the forefront of our mind. Unfortunately, rodent models do not recapitulate many EGFR-based skin toxicities, and biologics that bind human and not murine EGFR (as in our CT-12xx series as well as in cetuximab and panitumumab) require testing in NHPs in order to study skin effects. This testing is beyond the scope of this study; furthermore, we have opted to shift the revision away from extolling this series of molecules as clinical candidates in favor of emphasis on platform capabilities (see below response to Q3).

(3) Figure 8. The in vivo efficacy of CT-1212-1 is not convincing. Authors should compare the in vivo efficacy of the new drugs to Osimertinib, a drug used in the clinic for EGFR-mutant NSCLC (e.g., H1975). Long-term experiments, including tumor growth curves and mouse survival, are needed to show the efficacy of CT-1212-1 and/or CT-1222-1 in vivo. In addition to Ki67 and EGFR, authors should also analyze cell death markers and pEGFR/pERK. The toxicity of the drugs should also be evaluated. Finally, authors should include additional mouse models to confirm the major conclusion (e.g., tumor growth inhibition).

Since submission, we have conducted several in vivo efficacy studies with our molecules in the H1975 flank tumor model. There is a dose-dependent reduction in tumor growth; however, the effect is not so strong as to warrant further development at this time. This includes a decision to not test this series of molecules in an additional tumor model. As a result, we have re-written or re-phrased certain parts of the manuscript to de-emphasize the clinical potential of this specific series of EGFR-targeting molecules, and are instead emphasizing the potential for CYpHER as a platform. We believe the data presented in Figure 8 remains useful for demonstrating the ability of CYpHER molecules to act on a target in vivo and to provide adequate PK exposure. Moreover, our future work will be focused on CNS applications for this technology, as the combination of CNS transport capabilities (via TfR), and the fact that most CNS applications for TPD do not require the near-complete elimination of function that is often required in oncology applications, makes this a more promising avenue for near-term clinical applications.

We have included the phospho-EGFR Western blots in the revision. A combination of difficulty finding well-behaved antibodies, and a shift in focus away from pushing EGFR CYpHER as a clinical candidate (which would indeed merit investigation of downstream pathway activity), led us away from including other markers in favor of focusing on CYpHER's effects on EGFR itself.

Reviewer #2 (Remarks to the Author):

In this work, Nairn and colleagues developed a CYpHER technology that allows for durable lysosome-mediated degradation of target proteins in a catalytic manner. Compared with molecular glues and PROTACs that drive protein degradation catalytically, a key limitation for lysosomal pathway-dependent degraders is their perish together with the protein target, rendering them less durable in efficacy. Nairn et al. addressed this challenge by imparting the recycling behavior of Tf-TfR complex between the cell surface and endosomes back and forth to CypHERs. The concept of this work is interesting and such design may represent as an iteration for TPD drugs. While I acknowledge the novelty and comprehensive experimental design of this work, the unsatisfying quality of some data, the lack of statistical analysis throughout the manuscript, and concerns about biological reproducibility preclude me from recommending acceptation in its current form. Besides, more lines of evidence pertaining to the recycling characteristic of CYpHERs as well as investigation on their valency effect on protein degradation should be provided.

1.The authors described that CYpHER molecules were constructed on the basis of cystine-dense peptide (CDP) miniproteins. Explanations are needed to elucidate why CDPs were chosen as the building block? What properties do CDPs have?

The chosen TfR-binding CDP has several qualities that make it attractive for inclusion in CYpHER molecules. By being small and resistant to proteolysis, it is of low risk for immunogenicity. It is part of a well-characterized allelic series, permitting facile adjustment of affinity. We have previously co-crystalized it with TfR, giving us a deeper understanding of its interactions. Finally, it is cross-reactive with mouse, which facilitates in vivo studies without requiring introduction of a human TfR transgene. Some of this was in the manuscript already or in

cited, previous work, but we have emphasized certain aspects of it in the revision. Many of these same qualities are also true for the PD-L1-binding CDP. We will also note that CYpHER molecules neither depend on, nor exclusively use, CDPs for binding domains. The CT-12xx series uses VHH nanobodies for EGFR engagement.

2.I have to say most, if not all, statistical data presented in this manuscript lack significance analysis, which dampens readers' confidence on the validity of relevant results. In some cases (Fig. 1F), fold changes in each group should be marked for better comparison.

Statistical significance in comparisons have been included where lacking.

3.In Fig. 2B and 3D, is there any evidence or biological basis to illustrate that the GFP tag is localized outside endo/lysosomal cavity? If so, the GFP should not be degraded to show fluorescence decay.

Figure 3J demonstrates accumulation of EGFR-GFP signal in many intracellular compartments, which includes, but is not limited to, the lysosome. This is a fairly commonplace phenomenon in the eTPD literature, including the fact that signal from the fused fluorescent protein is not immediately quenched upon exposure to the lysosome.

4.The quality and resolution of confocal fluorescence images shown in Fig. 2C-E are poor (some images are blurry; all images lack scale bar). Please refine these data. In addition, illuminating the locations of lysosomes through a costaining assay would be more informative.

The images are not confocal, they are from a 20x objective fluorescent microscope taken at a single depth of focus, so refinement options are limited. We did substitute a somewhat less busy image for "Untreated" within panel C, and took another pass at contrast enhancement for all three of the panel C images, which may help. The images in panels D and E are as "un-blurry" as they can be made to be; they were captured at the focus depth with the sharpest edges / punctate staining, but with a single focal field, there will inevitably be some fuzzy areas. Trying to sharpen them in image analysis software would result in artifacts that we'd prefer not to introduce to the images.

Regarding co-staining the lysosomes, this was performed in Figure 3J, albeit for EGFR-GFP as opposed to PD-L1-GFP.

Scale bars were added to at least one image per set of equivalent magnification/scale images and referenced in the relevant figure legends.

5.I strongly recommend the authors to provide western blot results along with their flow cytometric data shown in Fig.2G,I,K, which is necessary to directly prove the degradation of total PDL1 proteins by CyPHERs.

We attempted Western Blotting of these samples but were unsuccessful. We have adjusted our phrasing in describing this data to clarify that protein elimination was as measured by flow.

6.What conclusion could be drawn from the comparison between CT-4212-1 (flexible linker) and CT-4121-3 (rigid linker)? There seems no instructive information pertaining to the design of linkers in CYpHER molecules.

The major differentiating factor between CT-4212-1 and CT-4212-3 has less to do with linker rigidity and more to do with the overall architecture. CT-4212-1 has the Fc in the middle, between the N-terminal PD-L1 binder and C-terminal TfR binder. CT-4212-3 has the Fc on the N-terminus with TfR-binder in the middle and PD-L1-binder on the C-terminus. This is useful as a demonstration that CYpHER molecules can use target-engagers that function best with either N- or C-terminal fusion to the scaffold; the PD-L1-binder seems to function well in either orientation, but our EGF variant (built into CT-5212-3) is predicted to work better fused via its N-terminus, a'la the PD-L1-binder in CT-4212-3. As for the rigidity of the linker in CT-4212-3, because the PD-L1-binder and TfR-binder are similar in structure and are CDPs, we wanted to reduce the risk of cross-oxidation during folding, and a rigid linker between the two reduces the risk of contacting one another prior and forming improper cystines. An abbreviated version of this explanation was added to the text.

7.What is the purpose to zoom in the HPLC spectrum in Fig. 3c?

In protein therapeutics, multimerization/aggregation, even at fairly subtle levels, can cause issues with immunogenicity and manufacturing, so we wanted to demonstrate the absence of even trace amounts of multimers in this prep.

8.The western blot result in Fig. 3F should be quantified to mark degradation efficiency.

This was added in the revised figure.

9.All microscopy images should be marked with scale bars.

This was done in the revision, albeit only once per group of images with identical scale and magnification.

10.It looks abnormal in Figure 3J that lysosomes, as illuminated by the CellLight Probe, are so sparse, with no more than three “dots” in each cell. The authors should explain this.

The lysosomes were lit up by a commercial baculovirus-delivered fluorescent protein. The infectivity of this reagent was relatively low.

11. I appreciate the well design of the catalytic uptake assay that demonstrated the catalytic mechanism of action for CYpHERs. However, more characterizations are needed to reveal this recycle process. For example, what is the decay kinetics for cell surface-retained CYpHER molecule? How long the degradation effect can be maintained after the withdrawal of CYpHERs?

Some more data to this effect has been added to the revision, found in Fig. S5.

12.In Fig. 4G, how to understand the rebound of EGFR expression in some cell lines after withdrawal? How to exclude the intrinsic difference across cell lines in their ability to recover EGFR expression to baseline? A control degrader without pH sensitivity will be helpful to address this issue.

Some of this can be better understood with the additional data added in Fig. S5, but it is inevitable that different cell lines will have different kinetics of EGFR rebound. We’ve clarified this point in the revised text.

13.It seems that monovalent degraders, e.g, 1211-1 and 1112-1, showed lower degradation efficiencies than divalent 1212-1 after withdrawal (Fig. 5B). Quantitative results of surface and total EGFR levels corresponding to Fig.5B should be provided. If so, the valency effect of CYpHERs on degradation efficiency should be carefully discussed.

Some additional data on surface EGFR levels upon treatment and/or withdrawal (now Fig. 5C) with the valence-modified CYpHERs was added in the revision, taking the place of the withdrawal images in Fig. 5B. This line (A549-EGFR-GFP) does not demonstrate total EGFR-GFP loss, as assessed by GFP quantitation; this has been noted in the revision. We have also made sure that the observation of total EGFR reduction in the 293T-EGFR-GFP cells was not phrased to imply that total EGFR reduction is always expected or seen in all cell lines.

14.Why the authors compartmentalized western blot results in Fig. 7B and C? Were those stripes in different groups obtained from the same gel and imaged under identical exposure settings? Uncropped original gel images should be provided for check. In addition, pEGFR levels in different groups after withdrawal should also be evaluated.

The uncropped original gel was included in the revised supplement. It was compartmentalized for easier viewing/interpretation, but they were all on the same blot. The effects of withdrawal on EGFR phosphorylation are interesting but we believe such an analysis would be better served in its own dedicated study, where additional factors like the dynamics of recycling saturation can be included; it would be included in future

studies should this series of molecules be further developed.

15.The authors designed five CYpHER formulations in the animal experiments, as shown in Figure 8A, but some key control groups, such as CT-1232-1/1211-1, are missing in the final efficacy evaluations (Figure 8E,G). In addition, why the degrader with rigid linker (5212-3) showed comparable degradation effect with its flexible linker counterpart, but has no effect on Ki67 inhibition?

For the final efficacy evaluations, we chose not to include CT-1232-1 (a negative control) because the experiment was a pilot experiment focused on looking for a pharmacodynamic response from the active agents; in this context, we decided that a vehicle control fills the role of a steady-state comparator, especially when we consider that CT-1232-1 has no in vitro effect on EGFR-GFP trafficking (Fig. 5C) and minimal effects on EGF-induced phosphorylation (Fig. 7B).

The lack of Ki67 inhibition by CT-5212-3 is indeed a curiosity. It may have to do with different biodistribution; the EGFR binder is different from that of the CT-12xx-1 series, and unlike the binder in the CT-12xx-1 series, it is cross-reactive with murine EGFR. It was also shown (Fig. 7C) that CT-1232-1 is less effective at suppressing EGF-induced EGFR phosphorylation than the CT-12xx-1 series. Either of these could plausibly blunt in vivo cell growth suppression.

Reviewer #3 (Remarks to the Author):

The authors described a very interesting system for a targeted protein degradation strategy which can be applied for targeting to tumors, including the ones localized in CNS.
The work is original and manuscript is well described and discussed. However, some points must be addressed especially in consideration of the applicability of the system to the clinic.

Major concerns:

The choice of the transferrin receptor as a capture molecule is rational because it is advantageous from the point of view of degradation of the target molecule, however it entails a series of implications that should be verified and discussed.

Neither in this work, nor in the previous one in which the specificity of the binding with TfR is demonstrated, there are data on the effect of this system on iron metabolism.
The authors demonstrate the biological effect of the treatment on the target molecule but do not demonstrate whether there is a side effect on iron metabolism. What effect does it have on erythropoiesis? If the system is selected to last over time, could this induce iron deficiency?

New data in the revision includes CBC data from a 4-week dose response experiment in animals with CT-1222-1. There was no apparent effect on reticulocyte or erythrocyte levels.

Transferrin Receptor 2 is homologous molecule of TfR1, it has a role in modulate iron homeostasis and loss of its function results in excess circulating iron and developed of a disorder called hemochromatosis. Has it been tested whether CYPHER also binds TfR2?

It is not included in this work, but the TfR binder does not cross-react with TfR2.

The lack of statistical analysis does not seem to make possible an accurate comparison of the data.

As detailed below, statistical analysis has been spruced up.

Minor point:

In the abstract it seems that the degradation system is valid in general while it refers to membrane proteins, it should be specified better.

We've tried to better clarify this in the revision.

*Continued review report:

The manuscript is a very interesting piece of work that demonstrates an innovative and potentially very useful therapeutic approach.

Major

Although the data presented are quite extensive and convincing, I believe it is essential to employ more statistical tests. Specifically:

- Fig. 3: Statistical analysis of H and I. Especially to see if the levels of Surface EGFR are statistically different in different intervals or if the value remains constant.

These statistics have been added to the revision. We will note that, because the data comes in the form of flow cytometry quantitation where the number of events tends to be in the thousands, it is easy for a subtle change to appear statistically significant; this is why we are soft on our messaging about whether an effect is notable or not, choosing to focus on those with objectively obvious differences.

- Fig. 4: Statistical analysis of B, C, E, F, G, and H. A more comprehensive statistical model, considering also the different cell lines, could provide valuable insights into a potential mechanism linking CYpHER activity to the number of TfR and/or EGFR receptors.

These statistics have been added to the revision. As for a more comprehensive statistical model, that would indeed be interesting but we believe such an analysis would be better served in its own dedicated study, where additional factors like the dynamics of recycling saturation can be included. We have, however, included in the supplement (New fig. S4) some plots of EGFR Reduction vs Baseline Surface EGFR or vs Baseline Surface TfR for the sake of more facile visualization of clear relationships (or lack thereof).

- Fig. 7: D-I. Due to large variance among initial points, when the concentration is practically 0, I deem it essential to conduct more detailed statistical analyses. The comparison with "standard" therapies is arguably one of the most important data points. Additionally, it is crucial to understand whether the decrease in cell levels is due to the internalization and degradation of EGFR or, for example, due to difficulties in the iron supply of these cells resulting from the decrease in TfR (as indicated in fig. S4).

We've included a table in the supplement with ANOVA comparisons of viability vs. CYpHER for each shared concentration. Regarding the effects of TfR disruption, we've determined that the in vitro cultures have an acute sensitivity to iron uptake disruption, as supplementation with a chelated iron (ferric ammonium citrate [FAC]) can suppress the growth inhibition in both lines where we tested, A549 and SW48 cells. This data, and a necessary re-write of the growth inhibition section to be clear about this confounder, has been added to the revision. Nevertheless, we also see in the A549 cells (a KRAS mutant line) that supplementation with EGF can provide a migratory phenotype and additional growth, and that EGFR CYpHER, but not a control molecule that only binds TfR, can suppress these EGF-induced phenotypes when FAC is present. This demonstrates that EGFR CYpHER can impact ligand-dependent growth, even in a line with a bypass mutation. This latter experiment is also in the revision.

Minor

- Fig. 6: Statistical analysis of C, D, and E.

This analysis is in the revision.

- Conduct statistical analyses of the graphs in fig. 2 (F-K). Additionally, it would be useful to perform quantitative analyses of images C-E.

The analysis of the graphs in Fig. 2, F-K are included in the revision. The cropped sections from the images in C-E were chosen to represent cells with exemplary EGFR localization but do not represent the wide range of cell GFP signals. They are also prone to saturation, which precludes quantitation; as the flow data demonstrates, it covers several logs of EGFR-GFP signal, making it non-trivial to set the gain on the microscope to be able to avoid saturation while still visualizing low-expressing cells. The quantitative flow analysis is meant to solve both of these issues: data from thousands of cells and a method with better dynamic range.

- Figure S4 shows fluctuations in TfR levels, which are labeled as "Mild" for some cells, which indeed have fewer TfR: however, it is not clear how it is possible that in some conditions (A549:1hr and H1975:3day) the number of TfR receptors is even higher than in untreated ones. My concern is that, rather than being caused by the process described by [47], it may be a variation that does not exist (a simple statistically insignificant variation). I would encourage the authors to conduct more in-depth statistical analyses and to better comment on the result.

Statistical comparisons were included in the revision, and we've altered our phrasing regarding these TfR changes to make it more clear that the effects on surface TfR can be considered to fall into one of two categories: objectively substantial reductions that persist up to 3 days and that rebound towards Untreated levels upon drug withdrawal (H358 and H1650), and responses that may represent drug-dependent effects on TfR expression and/or trafficking but are not persistent with continuous drug exposure (A549 and H1975).

Reviewer #4 (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 appreciate the time taken and the training provided. Very important, indeed.