

Decoding the Selective Chemical Modulation of CYP3A4

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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)

In this work, the authors identified non-suicide selective CYP3A4 inhibitors with novel chemical scaffold from an unbiased high-throughput screening (HTS) campaign and subsequent chemical analog design. The authors found that the differential positioning of the C-terminal loop between CYP3A4 and CYP3A5 would prevent these compounds from binding to CYP3A5 but not to CYP3A4 in a favorable conformation, thus leading to selective inhibition. Additionally, A unique surface within the ligand binding pocket of CYP3A4 was identified as essential for the preferential binding of these inhibitors. Overall, this work is well-organized and the experiments are well conducted, while the data are solid to support the conclusions. Thus, I recommend that this manuscript can be accepted after minor revision.

Revision suggestions as follows,

1. In Fig.1a, the authors should indicate where the screened compound libraries (9299 compounds) came from.
2. In the clustering analysis, were compounds used all tested compounds (9299 compounds), or 41 commercially available compounds with high potency against CYP3A4 and high selectivity? It was recommended that the authors should describe in detail.
3. In Fig.3, SCM-08 inhibited CYP3A4 and CYP3A5 at the recombinant enzyme level with IC₅₀ of 0.43 μ M and 20 μ M, respectively. The IC₅₀ values of SCM-08 for inhibiting CYP3A4 and CYP3A5 at the living cell level were 0.21 μ M and 9 μ M, respectively. The author should explain why the inhibitory effect of SCM-08 at the live cell level is stronger than at the recombinant enzyme level.
4. The test compounds were dissolved in DMSO. Excess DMSO will affect the enzymatic activity of CYPs, and the authors should indicate the content of DMSO in the CYP reaction system.
5. The authors tested the inhibitory effect of SCM-08 on CYP3A4 and CYP3A5 at the living cell level. The cytotoxicity of SCM-08 should be confirmed.
6. Line 464, 50 should be subscripted in IC₅₀.
7. The metabolic stability of SCM-08 should be supplemented.
8. Please explain the reason for selecting SCM-11 not SCM-07 for further chemical modifications.
9. SCM-25 exhibited selective inhibition on CYP3A4 in the final chemical modifications. It was recommended that the authors add the its inhibitory potencies against major human CYPs.

Reviewer #2

(Remarks to the Author)

The submitted article by Wang et al. titled Decoding the Selective Chemical Modulation of CYP3A4 details the research team's work identifying selective CYP3A4 inhibitors. Multiple analogs were made to explore the impact of structural changes on inhibition. The researchers have significant structural data to better understand the nature of the inhibition and guide the formation of future inhibitors. The described inhibitors may represent the best-characterized tool compounds for exploring the importance of CYP3A4 and CYP3A5 on the metabolism of compounds.

However, the current manuscript is written from the perspective that the selective CYP3A4 inhibitors could be co-dosed with compounds that are metabolized by CYP3A4 to increase oral absorption or extend their half-life. The researchers state that selectivity will decrease unintended drug interactions and improve safety. I believe this perspective is poorly reasoned, and there are minimal circumstances where a selective CYP3A4 inhibitor would be clinically superior to a pan-CYP3A inhibitor. There is a very high substrate overlap between CYP3A4 and CYP3A5, with only a handful of known drugs that show a high

degree of selectivity. One of the compounds that CYP3A5 more efficiently metabolizes is tacrolimus, and the researchers cite this as benefitting from a selective CYP3A4 inhibitor. Tacrolimus has a narrow therapeutic index, and the patient dose is adjusted to achieve the required drug level. On average, patients who express functionally active CYP3A5 require a higher dose than those who do not. A pan CYP3A inhibitor would elevate tacrolimus levels and be contradicted; however, given that over 50% of patients do not express active CYP3A5 and instead metabolize tacrolimus via the less efficient CYP3A4 pathway, selectively inhibiting CYP3A4 would still elevate tacrolimus levels in the majority of patients and would not be advised. Active CYP3A5 is expressed by approximately 50% of African Americans and 10% of Caucasians. A pan inhibitor such as ritonavir blocks both CYP3A4 and CYP3A5, and its impact can be predicted without patient genotyping data, which is unlikely to be available.

Reviewer #3

(Remarks to the Author)

This is a tour de force study on the discovery of inhibitors that are selective towards CYP3A4. The hypothesis on why some of these inhibitors don't bind so well to 3A5 is reasonable. Despite the quality of this work, there's not much novel or new about how these inhibitors work. There is a very large structural and functional database on 3A4 primarily by Sevrioukova et al, none of which was referenced, that has provided insights into conformational dynamics and what is required to make a good 3A4 inhibitor. Therefore, while this is solid work that has achieved the stated goal of finding more selective 3A4 inhibitors, the lack of major new insights beyond what has already been published precludes on a recommendation for publication in Nature Communications. I suggest the authors seek publication in a more specialized Journal. Perhaps J. Med. Chem.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The previous comments I raised are now addressed in appropriate way that I think the paper is acceptable as this revised version. Before publication, the authors need to pay attention to the following minor issues:

1. Line 14, "pan-CYP3A inhibitor" should be "pan-CYP3A inhibitors".
2. There should be a space between the number and the unit, for example, Line 132.
3. The time unit (hour/h) should be written in a uniform manner.

Reviewer #2

(Remarks to the Author)

The authors have addressed the points brought up in the review. Supplementary Fig 17 and Table 11 fit in well with the proposed benefit from a selective 3A4 inhibitor.

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We have addressed all the points raised by the reviewers (see below our point-by-point response). The revised manuscript text (with track changes) reflects all the changes made.

Reviewer #1 (Remarks to the Author):

In this work, the authors identified non-suicide selective CYP3A4 inhibitors with novel chemical scaffold from an unbiased high-throughput screening (HTS) campaign and subsequent chemical analog design. The authors found that the differential positioning of the C-terminal loop between CYP3A4 and CYP3A5 would prevent these compounds from binding to CYP3A5 but not to CYP3A4 in a favorable conformation, thus leading to selective inhibition. Additionally, A unique surface within the ligand binding pocket of CYP3A4 was identified as essential for the preferential binding of these inhibitors. Overall, this work is well-organized and the experiments are well conducted, while the data are solid to support the conclusions. Thus, I recommend that this manuscript can be accepted after minor revision.

Response: Thank you for your constructive comments. We have included our point-by-point response below.

Revision suggestions as follows,

1. In Fig. 1a, the authors should indicate where the screened compound libraries (9299 compounds) came from.

Response: The compound library is a subset of St. Jude in-house library acquired from several commercial sources (mostly from ChemBridge, Enamine, ChemDiv, Life Chemicals, Otava and InterBio) with molecular descriptors that conformed to drug-like physicochemical characteristics while minimizing pan-assay interference compounds (PAINs). We have also modified the same statement in the Methods section, lines 496-497 of the main manuscript.

A more detailed description of the St. Jude in-house library can be found in the following publication: Nishiguchi, G. *et al.* (2021) *Drug discovery today*, 26(4), 1060-1069 (reference 24 in our manuscript).

2. In the clustering analysis, were compounds used all tested compounds (9299 compounds), or 41 commercially available compounds with high potency against CYP3A4 and high selectivity? It was recommended that the authors should describe in detail.

Response: Thanks for the suggestion. The analysis was made using 956 compounds selected from primary screening based on a cutoff of $\geq 80\%$ inhibition (line 75 of the main manuscript). We have added the missing information in the Fig 1b legend:

“Clustering by structural similarities of the 956 compounds selected for dose–response studies against CYP3A4 and CYP3A5, with the three lead selective CYP3A4 inhibitors indicated as blue, green and pink.”

3. In Fig.3, SCM-08 inhibited CYP3A4 and CYP3A5 at the recombinant enzyme level with IC₅₀ of 0.43 μ M and 20 μ M, respectively. The IC₅₀ values of SCM-08 for inhibiting CYP3A4 and CYP3A5 at the living cell level were 0.21 μ M and 9 μ M, respectively. The author should explain why the inhibitory effect of SCM-08 at the live cell level is stronger than at the recombinant enzyme level.

Response: We did notice the stronger inhibition by SCM-08 in the cell-based assay compared to the biochemical assay. This may be caused by several factors, such as the relatively lower CYP protein concentration in cells compared to that in the biochemical assays, or differences in cofactor concentrations (e.g., NADPH, P450 reductase, cytochrome b5). It is difficult to determine the exact reason for the stronger inhibitory potency in cells. Nevertheless, the CYP3A4/CYP3A5 selectivity ratio remains the same in cell-based and biochemical assays.

4. The test compounds were dissolved in DMSO. Excess DMSO will affect the enzymatic activity of CYPs, and the authors should indicate the content of DMSO in the CYP reaction system.

Response: The DMSO concentration in both biochemical and cell-based assays is 0.25%, which is within the DMSO tolerance suggested by the Promega P450-Glo assay manual for Luciferin-IPA (*“CYP3A4 reactions with Luciferin-PPXE and Luciferin-IPA show little or no sensitivity to DMSO at or below 0.25%”*). We have added the missing DMSO concentration information in the Methods section, lines 522, 552, 566 of the main manuscript.

5. The authors tested the inhibitory effect of SCM-08 on CYP3A4 and CYP3A5 at the living cell level. The cytotoxicity of SCM-08 should be confirmed.

Response: Thanks for the suggestion. The cytotoxicity assay showed that SCM-08 is not toxic up to 60 μ M for 72 hours in 293T/17 cells, which is the cell line used for all cell-based assays (see new Supplementary Figure 16d). The toxicity of SCM-08 is also lower than that of currently prescribed non-selective inhibitor, ritonavir.

We have also modified lines 381-382 of the main manuscript: *“The compound also remains selective for CYP3A4 in cell-based assays and is not cytotoxic up to 60 μ M (Supplementary Fig. 16c-d).”*

6. Line 464, 50 should be subscripted in IC₅₀.

Response: We have changed it to the subscripted IC₅₀ (line 503 of the revised main manuscript)

7. The metabolic stability of SCM-08 should be supplemented.

Response: Thanks for the suggestion. We tested the metabolic stability of SCM-08 in human liver microsome. The compound has a half-life ($T_{1/2}$) of 0.99 ± 0.05 hour at 1 μ M and >2 hours at 5 μ M, which is similar to ritonavir (see new Supplementary Table 10). The longer half-life at higher compound concentrations is expected because enzymes may be saturated at higher compound concentrations, as observed with the control compound verapamil.

We have added the following sentence in lines 384-386 of the main manuscript: *“Additionally, the metabolic stability of SCM-08 in human liver microsomes is similar to that of ritonavir, with half-lives of 0.99 ± 0.05 hour at 1 μ M and >2 hours at 5 μ M (Supplementary Table 10).”*

8. Please explain the reason for selecting SCM-11 not SCM-07 for further chemical modifications.

Response: We agree that SCM-07 is also a promising starting point, and indeed this led to a more selective CYP3A4 inhibitor, SCM-08, that was highlighted in the manuscript. However, as stated in lines 261-263 of the main manuscript, the two most promising SCM-07 analogs that have no CYP3A5 inhibition (SCM-10 and 14) are mechanism-based inhibitors that lost selectivity upon pre-incubation (see Supplementary Figure 9). Therefore, we decided to make further chemical modifications based on the non-mechanism-based inhibitor SCM-11, as its chlorophenyl moiety is resistant to metabolism while providing good selectivity for CYP3A4.

9. SCM-25 exhibited selective inhibition on CYP3A4 in the final chemical modifications. It was recommended that the authors add the its inhibitory potencies against major human CYPs.

Response: Thank for the suggestion. Our assay results showed that SCM-25, like SCM-01 and 08, is selective for CYP3A4 against other major human CYP enzymes, which means this compound can be another starting point for developing selective CYP3A4 inhibitors. We have added its test results in the Supplementary Table 9.

Reviewer #2 (Remarks to the Author):

The submitted article by Wang et al. titled Decoding the Selective Chemical Modulation of CYP3A4 details the research team's work identifying selective CYP3A4 inhibitors. Multiple analogs were made to explore the impact of structural changes on inhibition. The researchers have significant structural data to better understand the nature of the inhibition and guide the formation of future inhibitors. The described inhibitors may represent the best-characterized tool compounds for exploring the importance of CYP3A4 and CYP3A5 on the metabolism of compounds.

However, the current manuscript is written from the perspective that the selective CYP3A4 inhibitors could be co-dosed with compounds that are metabolized by CYP3A4 to increase oral absorption or extend their half-life. The researchers state that selectivity will decrease unintended drug interactions and improve safety. I believe this perspective is poorly reasoned, and there are minimal circumstances where a selective CYP3A4 inhibitor would be clinically superior to a pan-CYP3A inhibitor. There is a very high substrate overlap between CYP3A4 and CYP3A5, with only a handful of known drugs that show a high degree of selectivity. One of the compounds that CYP3A5 more efficiently metabolizes is tacrolimus, and the researchers cite this as benefitting from a selective CYP3A4 inhibitor. Tacrolimus has a narrow therapeutic index, and the patient dose is adjusted to achieve the required drug level. On average, patients who express functionally active CYP3A5 require a higher dose than those who do not. A pan CYP3A inhibitor would elevate tacrolimus levels and be contradicted; however, given that over 50% of patients do not express active CYP3A5 and instead metabolize tacrolimus via the less efficient CYP3A4 pathway, selectively inhibiting CYP3A4 would still elevate tacrolimus levels in the majority of patients and would not be advised. Active CYP3A5 is expressed by approximately 50% of African Americans and 10% of Caucasians. A pan inhibitor such as ritonavir blocks both CYP3A4 and CYP3A5, and its impact can be predicted without patient genotyping data, which is unlikely to be available.

Response: Thank you for your positive comments on our novel compounds, and we appreciate the insights provided regarding the overall value in the development of selective CYP3A4 inhibitors.

A significant proportion of the global population express CYP3A5 (according to the study by Zhou *et al.* [reference 19 in our manuscript], 5.3% of Europeans, 56.3% of Africans, 27.7% of East Asians, 33.2% of South Asians, and 17.5% of admixed Americans are CYP3A5 expressors). The concerning role of CYP3A5 in drug-drug interactions has been increasing as growing number of people are subjected to polypharmacy, whereby multiple medications are taken concurrently. Additionally, CYP3A5 inhibition can result in dangerously elevated plasma levels of certain drugs due to reduced clearance. Reports in the literature indicate several drugs that are preferentially metabolized by either CYP3A4 or CYP3A5 (reference 13 in our manuscript), and for the current revised manuscript, we expanded the list of selective CYP3A4/5 substrates by evaluating 1491 FDA-approved drugs by mass spectrometry. It is therefore conceivable that the number of drugs that are selectively metabolized by either CYP3A paralog will increase in the future, and we believe that selective CYP3A4 inhibitors can play a notable role in such circumstances.

Based on the valuable perspective provided by the reviewer, we enhanced the value of our manuscript by expanding the rationale for the need of selective CYP3A4 inhibitors as reflected in the introduction section (lines 37-49), discussion section (lines 408-412), new Supplementary Fig. 17, and new Supplementary Table 11. We hope the reviewer welcomes our contributions regarding the design and mechanistic understanding of selective CYP3A4 inhibitors as an additional arsenal in drug discovery, a challenging task due to the high functional and structural similarities between CYP3A4 and CYP3A5.

Reviewer #3 (Remarks to the Author):

This is a tour de force study on the discovery of inhibitors that are selective towards CYP3A4. The hypothesis on why some of these inhibitors don't bind so well to 3A5 is reasonable. Despite the quality of this work, there's not much novel or new about how these inhibitors work. There is a very large structural and functional database on 3A4 primarily by Sevrioukova et al, none of which was referenced, that has provided insights into conformational dynamics and what is required to make a good 3A4 inhibitor. Therefore, while this is solid work that has achieved the stated goal of finding more selective 3A4 inhibitors, the lack of major new insights beyond what has already been published precludes on a recommendation for publication in Nature Communications. I suggest the authors seek publication in a more specialized Journal. Perhaps J. Med. Chem.

Response: We thank the reviewer for the positive comments on the quality of our work and appreciate the reviewer's insightful comments. We agree with the reviewer of the outstanding body of work in the literature by Sevrioukova and others towards the design of potent CYP3A4 inhibitors. Our contributions differ from previous publications as we focus on the challenging objective of selectively inhibiting CYP3A4 while minimizing the inhibition of the highly homologous CYP3A5, and understanding the basis of such selectivity based on extensive structural studies. The selective CYP3A4 inhibitors we developed represent a distinct class of compounds among all CYP3A4 ligands crystalized to date (see Supplementary 18 and lines 437-439 of the main manuscript).

The unique and significant aspect of our work is to emphasize the selective inhibition of CYP3A4 compared to CYP3A5 based on a series of chemical analogs of SCM-01. Importantly, we uncovered the structural features that describe such selectivity based on 10 co-crystal structures of CYP3A4/5 with SCM-01 analogs, including a direct comparison of the structures of CYP3A4 and CYP3A5 in complex with SCM-27 (new data that we include in the revised manuscript – see Supplementary Fig. 14f-h and lines 351-360 of the main manuscript). To our knowledge, no prior study has disclosed the structural mechanism of selective CYP3A4 inhibition. Overall structural comparisons between CYP3A4 and CYP3A5 have been reported showing several differences, such as the presence or absence of what we define as “surface 1” and dynamics of the C-terminal loop orientation. Our work provides experimental evidence that such observations can be applied in the design of CYP3A4 selective inhibitors, albeit with difficulties as we observed that small changes in the chemical structure of SCM-01 analogs can drastically affect affinity and/or selectivity. We believe that our manuscript provides a unique perspective to the field of cytochrome P450 and its impact on drug discovery.

We are also grateful to the reviewer for enhancing the value of our manuscript by incorporating references to important work by Sevrioukova and others, as reflected in our revised main manuscript:

Lines 235-236: Added sentence “*This surface has also been reported to play an important role in CYP3A4 binding by ritonavir and its analogs.*”⁴⁰⁻⁴²

Lines 420-421: Added sentences “*The structural difference between CYP3A4 and CYP3A5 C-terminal loop has been noticed previously.*”^{20,53} *Our work provides experimental evidence that such differences can be exploited in the selective inhibition of CYP3A4.*”

Lines 430-432: Modified the sentence “*Surface 1 found in CYP3A4 is replaced by a wall in CYP3A5 due to the position of the C-terminal loop, which forces the region A group of the analogs to interact with an alternative space (surface 2) that has been shown to be important for ligand binding in CYP3A4.*”⁴⁰⁻⁴²

We again thank the reviewers for their evaluation of our manuscript. We have made minor text edits to address the final comments of Reviewer 1, and our specific responses are written below in green text.

Reviewer #1:

The previous comments I raised are now addressed in appropriate way that I think the paper is acceptable as this revised version. Before publication, the authors need to pay attention to the following minor issues:

1. Line 14, “pan-CYP3A inhibitor” should be “pan-CYP3A inhibitors”.
 - We have changed “inhibitor” to “inhibitors”.
2. There should be a space between the number and the unit, for example, Line 132.
 - We have added the space.
3. The time unit (hour/h) should be written in a uniform manner.
 - We have now used “minute” and “hour” throughout the manuscript in a uniform manner.

Reviewer #2:

The authors have addressed the points brought up in the review. Supplementary Fig 17 and Table 11 fit in well with the proposed benefit from a selective 3A4 inhibitor.