nature portfolio

Peer Review File

Sol-moiety: Discovery of a water-soluble prodrug technology for enhanced oral bioavailability of insoluble therapeutics

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

In this manuscript, the authors developed a water-soluble prodrug technology, Sol-moiety, to promote the oral bioavailability of highly insoluble small molecule drugs. To test whether this technique can be applied in a wide range of drugs, a series of sol-moiety-drug conjugates were designed and synthesized. The results showed that these conjugates could remain steadily in stimulated gastric and intestinal environment, and provide good exposure levels in vivo via oral gavage. Adjusting the structure of prodrug could alter the pharmacokinetics and improve bioavailability. Finally, the sol-paclitaxel treatment achieved a 4-6-fold improvement in paclitaxel oral bioavailability, confirming that an oral delivery of paclitaxel could be transformative and this sol-moiety technology has the potential to solve the problem of orally ingested insoluble drugs. I recommend this work could be published in Nature Communications. There are some points needed to be clarified.

1. In the introduction, the authors choose to connect the phosphate position to the ortho-position both for synthetic ease and to create a sterically encumbered environment around the adjacent methylene group. Could the authors explain the reason for creating a sterically encumbered environment around the adjacent methylene group?
2. The authors measured 8 prodrug Papp values to support that the rate of sol-moiety hydrolysis will determine the concentration of liberated drug at the cell surface, effectively controlling the overall rate of drug absorption. However different parent drug permeation rate could also affect the prodrug Papp value. The Papp value could not indicate a positive correlation between the rate of hydrolysis and absorption. The data related to the hydrolysis rate of the prodrug need to be supplemented to provide further proof.
3. In Caco2 assay, the Papp value of 2i released drug is not detected, but in PK study, the AUC of 2i is 74.7 μM•hr which is the third highest in all groups. These two data seem to be contradictory. I would think that the sol-moiety conjugates might be observed in mouse plasma. Do the authors have a justification or explanation for that?
4. The authors mention a slight delay in drug release by the methyl and fluoro substituted analogs. However, Tmax could not align with drug release rate. The hydrolysis rate of sol-moiety (1i,1iii,1iv and 1v) and oral bioavailability need to be provided to further confirm the

hypothesis.

5. The authors mention the Tmax and T1/2 of 2i were longer than 2vi which may be due to the worse solubility of 2i in the gastrointestinal tract. Please add the solubility data to validate that.

6. The bioavailability of 2iv is shown in Figure 7b, but the 2vi is tested in the article. Please check for spelling mistakes.

Reviewer #2 (Remarks to the Author):

I reviewed this manuscript with great interest because there is a significant need for novel and widely applicable prodrug strategies. The phosphate prodrug strategy has been one of the most successful in improving solubility and dissolution rates, consequently enhancing oral absorption. However, the use of phosphate ester prodrugs is primarily limited to high-dose BCS Class II drugs, which are characterized by high permeability and low solubility. Any extension to this rather limited strategy, such as the described "Sol-moiety" strategy, if effective, is needed. Additionally, strategies beyond the typical phosphate or phosphonooxymethyl groups are also appealing from an IP perspective.

Despite certain weaknesses in the manuscript, such as the neglect of relevant literature discussed below, I find the manuscript highly interesting.

I have the following remarks regarding the manuscript:

As mentioned above, phosphate ester prodrugs have been very successful in producing marketed water-soluble prodrugs. However, this strategy is most useful when applied to drugs with specific properties—namely, high permeability and poor solubility. While rapid precipitation of the released poorly soluble parent drug is certainly a drawback with highly soluble prodrugs, this issue often comes from the poor selection of the drug for prodrugging. This has been described, for example, in Tycho Heimbach's publication (cited in the manuscript, reference #23). Additionally, an even more significant drawback is that phosphates are not applicable to every functional group due to slow bioconversion. For example, drugs with acidic NH groups tend to be released too slowly even from phosphonooxymethyl groups (see, for example, Guarino et al., Bioorg Med Chem Lett 17: 4910-13, 2007, and subsequent publications on sulfenamide prodrugs). Taking these more

significant drawbacks into account, the introduction is rather weak and narrowly focuses on only one specific concern with the existing water-soluble prodrug strategies.

Secondly, the authors failed to address the publication by Liu et al. from BMS, which described a quite similar prodrug strategy to the one in this manuscript (see Liu et al., J Med Chem 58: 7775-84, 2015). Even though Liu's paper does not specifically aim to reduce the hydrolysis rate, their goal was to achieve a suitable hydrolysis rate for improved oral absorption.

Thirdly, while these prodrugs were designed as water-soluble prodrugs, the manuscript lacks detailed solubility data and descriptions of the experimental setup. Additionally, one of the aims of the "Sol-moiety" strategy was to tune the hydrolysis rate of the released parent drug. From a prodrug design perspective, detailed hydrolysis data is now missing. It is important to know how rapidly the prodrugs undergo alkaline hydrolysis in the presence of intestinal S9 or pure alkaline phosphatase, and consequently, what the rates of appearance of the parent drugs are. This information might shed light on the surprising finding regarding the improved absorption of the two BCS class IV drugs.

Fourthly, the surprising finding of improved oral absorption of the two BCS class IV drugs with the water-soluble prodrug strategy needs further elaboration, as this is not typically, if ever, seen with phosphate or phosphonooxymethyl esters. Is the rate of hydrolysis the determining factor? What are these rates? Are there any other factors that make difference?

Finally, I understand that it is a lot to ask, but it would have been highly interesting to compare compound 1i to a more straightforward example, such as a phosphonooxymethyl prodrug.

More specific remarks:

1. From a prodrug design point of view, it would be important to point out in the abstract that the "Sol-moiety strategy" was tested with various functional groups.
2. I am not sure about the terminology used for "formyl phosphate." In prodrug literature,

this group is generally referred to as the “phosphonooxymethyl” group.

3. Introduction: Isavuconazonium sulfate is also a water-soluble prodrug in clinical use for both oral and IV administration. Please add this to the manuscript.
4. In vivo PK chapter: Remove the following: “…perhaps should be reclassified as a BCS class II molecule (poor solubility and good permeability).” The prodrug is hydrolyzed before absorption, and therefore, it is pointless to speculate about the classification of the prodrug or the released parent drug.
5. Detailed solubility data is missing for water-soluble prodrugs! Also experimental setup.

Figures:
1. Fig. 1A and 1B: Why are not all prodrugs drawn in their salt forms? I didn’t check, but more of these prodrugs are salts in clinical use and not only those drawn in the figures.
2. Fig. 1B: Use blue color for the phosphonooxymethyl group in fosphenytoin.

Jarkko Rautio
Professor
University of Eastern Finland

Response to Reviewers

Reviewer #1 (Remarks to the Author):

1. In the introduction, the authors choose to connect the phosphate position to the ortho-position both for synthetic ease and to create a sterically encumbered environment around the adjacent methylene group. Could the authors explain the reason for creating a sterically encumbered environment around the adjacent methylene group?

Response: Our reasoning was that the ortho position is more sterically encumbered than the para-position and the rate of hydrolysis would be slower. We based our assumption on Piizzi's (Reference 26) study of fluorogenic substrates of the alkaline phosphatase, PTP1B. Herein, they showed that an increase in steric bulk adjacent to a phosphate group significantly impaired the kinetics of hydrolysis (Kcat 13.3 to 0.002 and 0.005 S^{-1}) following addition of an OMe and Cl substituent respectively. We thank the reviewer for their comment and have reworded this sentence to provide clarity for the reader.

2. The authors measured 8 prodrug Papp values to support that the rate of sol-moiety hydrolysis will determine the concentration of liberated drug at the cell surface, effectively controlling the overall rate of drug absorption. However different parent drug permeation rate could also affect the prodrug Papp value. The Papp value could not indicate a positive correlation between the rate of hydrolysis and absorption. The data related to the hydrolysis rate of the prodrug need to be supplemented to provide further proof.

Response: We agree with the reviewer and have added the hydrolysis data for the Sol-moiety drug conjugates to Figure 4a and have reworded the data interpretation to provide clarity.

3. In Caco2 assay, the Papp value of 2i released drug is not detected, but in PK study, the AUC of 2i is 74.7 μM•hr which is the third highest in all groups. These two data seem to be contradictory. I would think that the sol-moiety conjugates might be observed in mouse plasma. Do the authors have a justification or explanation for that?

Response: We repeated the Caco-2 assay for compounds **2i** and **4i** (n = 2) and have updated the table with the observed P_{app} values. So far; we have looked but not observed any of the Sol-moiety-drug conjugates in mouse plasma at the dose levels we have currently used (75 mg/kg being the highest). We believe this is due to the strong ionic character associated with the Sol-moiety, especially those possessing the phosphonate group.

4. The authors mention a slight delay in drug release by the methyl and fluoro substituted analogs. However, Tmax could not align with drug release rate. The hydrolysis rate of sol-moiety (1i,1iii,1iv and 1v) and oral bioavailability need to be provided to further confirm the hypothesis.

Response: We thank the reviewer for their comment and have added the rate of hydrolysis to the table in Fig. 6. The methyl and methoxy substitutes had a lower rate of hydrolysis relative to fluoro and unsubstituted analogs. The Tmax values do not correlate to the rate of hydrolysis but that is in part to the small number of mice in each dose group and the frequency of blood draws.

5. The authors mention the Tmax and T1/2 of 2i were longer than 2vi which may be due to the worse solubility of 2i in the gastrointestinal tract. Please add the solubility data to validate that.
Response: *The solubility data on all compounds measured in SGF and HBSS has been added to the supplemental information (Table 2). There is a 7-fold difference in solubility between 2i and 2vi at pH 1.2 but both compounds are soluble at pH 6.5. We have updated the discussion with the manuscript accordingly.*

6. The bioavailability of 2iv is shown in Figure 7b, but the 2vi is tested in the article. Please check for spelling mistakes.
Response: We thank the reviewer for this observation and apologize for the confusion. Fig. 7b has been corrected to display 2vi.

Reviewer #2 (Remarks to the Author):

I reviewed this manuscript with great interest because there is a significant need for novel and widely applicable prodrug strategies. The phosphate prodrug strategy has been one of the most successful in improving solubility and dissolution rates, consequently enhancing oral absorption. However, the use of phosphate ester prodrugs is primarily limited to high-dose BCS Class II drugs, which are characterized by high permeability and low solubility. Any extension to this rather limited strategy, such as the described "Sol-moiety" strategy, if effective, is needed. Additionally, strategies beyond the typical phosphate or phosphonooxymethyl groups are also appealing from an IP perspective.
Despite certain weaknesses in the manuscript, such as the neglect of relevant literature discussed below, I find the manuscript highly interesting.

I have the following remarks regarding the manuscript:
As mentioned above, phosphate ester prodrugs have been very successful in producing marketed water-soluble prodrugs. However, this strategy is most useful when applied to drugs with specific properties—namely, high permeability and poor solubility. While rapid precipitation of the released poorly soluble parent drug is certainly a drawback with highly soluble prodrugs, this issue often comes from the poor selection of the drug for prodrugging. This has been described, for example, in Tycho Heimbach's publication (cited in the manuscript, reference #23). Additionally, an even more significant drawback is that phosphates are not applicable to every functional group due to slow bioconversion. For example, drugs with acidic NH groups tend to be released too slowly even from phosphonooxymethyl groups (see, for example, Guarino et al., Bioorg Med Chem Lett 17: 4910-13, 2007, and subsequent publications on sulfenamide prodrugs). Taking these more significant drawbacks into account, the introduction is rather weak and narrowly focuses on only one specific concern with the existing water-soluble prodrug strategies.

Response: We thank the reviewer for sharing their deep knowledge on the subject matter. The introduction was deliberately focused on the challenges we have experienced with utilizing prodrug technologies that are clinically approved. It was not our intent to perform a comprehensive literature review on alternative prodrug technologies that have been used preclinically (there are many excellent reviews that cover this subject). We are aware of the work of Guarino but our focus is on water-soluble prodrugs that undergo bioconversion to release the parent drug in the lumen and not following cellular absorption. We have added to the introduction and hope it is acceptable to the reviewer.

Secondly, the authors failed to address the publication by Liu et al. from BMS, which described a quite similar prodrug strategy to the one in this manuscript (see Liu et al., J Med Chem 58: 7775-84, 2015). Even though Liu's paper does not specifically aim to reduce the hydrolysis rate, their goal was to achieve a suitable hydrolysis rate for improved oral absorption.

Response: We thank the reviewer for bringing to our attention the work of Liu et al. We now reference this paper in our introduction as they were able to improve acid stability relative to the phosphonooxymethyl ether prodrugs.

Thirdly, while these prodrugs were designed as water-soluble prodrugs, the manuscript lacks detailed solubility data and descriptions of the experimental setup. Additionally, one of the aims of the "Sol-moiety" strategy was to tune the hydrolysis rate of the released parent drug. From a prodrug design perspective, detailed hydrolysis data is now missing. It is important to know how rapidly the prodrugs undergo alkaline hydrolysis in the presence of intestinal S9 or pure alkaline phosphatase, and consequently, what the rates of appearance of the parent drugs are. This information might shed light on the surprising finding regarding the improved absorption of the two BCS class IV drugs.

Response: We thank the reviewer for their comments and have added the hydrolysis data using purified human placental alkaline phosphatase (Fig 4, Fig 6 and supplementary information Table 3) as well as the solubility data for all compounds at pH 1.2 and 6.5 (supplementary information Table 2).

Fourthly, the surprising finding of improved oral absorption of the two BCS class IV drugs with the water-soluble prodrug strategy needs further elaboration, as this is not typically, if ever, seen with phosphate or phosphonooxymethyl esters. Is the rate of hydrolysis the determining factor? What are these rates? Are there any other factors that make difference?

Response: We thank the reviewer for their comments and have provided the requested rate of hydrolysis along with the solubility data for all compounds. There's a notable difference in solubility at pH 6.5 that we believe is influencing the improved oral bioavailability of Sol-paclitaxel **8vi** over **8i**.

Finally, I understand that it is a lot to ask, but it would have been highly interesting to compare compound 1i to a more straightforward example, such as a phosphonooxymethyl prodrug.

Response: We completely agree with the reviewer and had attempted to synthesize the phosphonooxymethyl prodrug of enzalutamide on several occasions. Unfortunately, we were unable to isolate the desired product under the reaction conditions we used (decomposition). However, we do compare the phosphonooxymethyl prodrug of paclitaxel **8vii** with the Sol-paclitaxel analogs **8i** and **8vi** and hope the reviewer finds this comparison to be interesting.

More specific remarks:
1. From a prodrug design point of view, it would be important to point out in the abstract that the "Sol-moiety strategy" was tested with various functional groups.
Response: We thank the reviewer for this comment and have updated the abstract to include "various functional groups".

2. I am not sure about the terminology used for "formyl phosphate." In prodrug literature, this group is generally referred to as the "phosphonooxymethyl" group.
Response: We thank the reviewer for this comment and updated the nomenclature in the manuscript.

3. Introduction: Isavuconazonium sulfate is also a water-soluble prodrug in clinical use for both oral and IV administration. Please add this to the manuscript.
Response: Isavuconazonium sulfate has been added to the introduction and Fig. 1a.

4. In vivo PK chapter: Remove the following: "…perhaps should be reclassified as a BCS class II molecule (poor solubility and good permeability)." The prodrug is hydrolyzed before absorption, and therefore, it is pointless to speculate about the classification of the prodrug or the released parent drug.
Response: This has been removed this statement from the manuscript.

5. Detailed solubility data is missing for water-soluble prodrugs! Also experimental setup.
Response: The solubility data has been added along with the protocol to the supplementary information section.

Figures:
1. Fig. 1A and 1B: Why are not all prodrugs drawn in their salt forms? I didn't check, but more of these prodrugs are salts in clinical use and not only those drawn in the figures.
Response: We thank the reviewer for bringing this to our attention. The figures have been corrected to include all salt forms.
2. Fig. 1B: Use blue color for the phosphonooxymethyl group in fosphenytoin.
Response: This has been corrected.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

My comments have been fully addressed. I recommend accept.

Reviewer #2 (Remarks to the Author):

The revised manuscript has successfully addressed all the remarks raised during the initial review process. I have only two very minor suggestions for the revised version.

First, you may want to rephrase the title of Figure 1, as BMS-751324 is not yet a commercial drug.

Secondly, please also include the half-lives of the disappearance of the prodrugs in Table 3 (Supplementary Information), as these are more informative for prodrug scientists than the drug formation rates expressed in pmol/min.

Response to Reviewers

Reviewer #2 (Remarks to the Author):

1. The revised manuscript has successfully addressed all the remarks raised during the initial review process. I have only two very minor suggestions for the revised version.
First, you may want to rephrase the title of Figure 1, as BMS-751324 is not yet a commercial drug.
Secondly, please also include the half-lives of the disappearance of the prodrugs in Table 3 (Supplementary Information), as these are more informative for prodrug scientists than the drug formation rates expressed in pmol/min.

Response: We have corrected the title to Figure 1a to read: Orally administered therapeutics that possess a water-soluble promoiety (e.g. phosphate or phosphonooxymethyl group).

Since this is an enzyme reaction ($d[P]/dt = kcat[S]/(Km + [S])$, not a first order process ($d[P]/dt = k[S]$), it is not appropriate to calculate half-lives when it is unknown if the [S] is in excess of Km. The kinetic rates of hydrolysis were determined using the linear portion of the plotted regression curve and provides an accurate differentiation between the substrates.