nature portfolio

Peer Review File

Intradermal Delivery of Lipophilic siRNAs Enables Prolonged Skin Retention and Sustained Gene Silencing in a Porcine Model

Corresponding Author: Dr Julia Alterman

Parts of this Peer Review File have been redacted as indicated to remove third-party material.

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)
This a nicely conceived study demonstrating that intradermally administered siRNAS can provide proprolonged silencing of the Jak1gene which has downstream effects on inflammtory cytokines and chemokines. Overall this study has important implications for treating dermal inflammatory conditions like psoraiasis and eczema. The only shortcoming is that data feom scrambled or other control siRNAS seem to be missing.

Reviewer #2

(Remarks to the Author)
The manuscript presents a lipophilic siRNA conjugate strategy for treating skin diseases. The authors provide favorable safety and PK/ PD profile for administering siRNA in a localized manner without significant systemic drug exposure. The data presented across large animal model and clinically relevant human skin tissue is very encouraging. The overall study is well-planned and executed and presented in a clear and systematic manner, however, there are certain aspects of the work which need additional experimentation and clarification:

1. Why did D-JAK13033 display considerable variability in the epidermal layer? Provide more explanation. Would increasing the sample size resolve the statistical significance?

2. It is recommended to include non-targeting (scrambled) siRNA control in the in vivo porcine experiment to showcase differences in inflammatory markers. Since the siRNA is targeting a protein involved in an inflammatory cascade, there could be some unforeseen changes in the baseline levels which may not be accurately captured from untreated and PBS-treated study groups. This is evident from supplemental Figure S2, where PBS-treated human biopsy samples appear to have lower JAK1 mRNA levels compared to the non-targeting control group.

3. What does the significance between PBS and on-target JAK1 siRNA look like in Figure S2?

4. The authors report more than 8 weeks of siRNA retention in porcine tissue after intradermal injection. However, for the efficacy studies, they selected 1 month as the end-point. Why was this timeline chosen? Additionally, is there a siRNA threshold concentration needed at the target site to achieve therapeutic benefit?

5. Make sure the graph axis is consistent across different figure panels for better visualization. For instance, in Fig. 3b, y-axis for CXCL levels in dermis vs. epidermis has different axis points.

6. Given the translational potential of this technology and the mention of an on-going Phase 2a human clinical trial, the authors should add a discussion about other delivery systems (transdermal patches, ointments, and so on) that may be utilized as an alternative to painful intradermal injections where the intended treatment surface area is larger.

Reviewer #3

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This manuscript leverages a less commonly studied porcine model alongside a widely used ex vivo model, providing a valuable comparative perspective. The authors adeptly integrate lipid chemistry and RNA chemistry to enhance gene silencing efficacy and tissue retention in the skin, which is the body's largest organ. Furthermore, the analysis of unconjugated, dendrimer-conjugated, and their docosanoic siRNA conjugates allow for meaningful comparison and context within the field. Notably, the authors report increased silencing in JAK1 for their leading siRNA conjugate in both human skin and the porcine model.
This work makes important advances in intradermal delivery technology. The inclusion of comparative data across different siRNA conjugates, presented in a more human-compatible porcine model, provides novel insight and may inform future research directions.

In addition, the manuscript addresses a central challenge in the field: extrahepatic delivery. Building on previous research, the authors progress this area toward greater clinical relevance, as evidenced by the ongoing Phase 2a human clinical trial of the leading compound, DCA-JAK1. The manuscript is compelling in its broad inclusion of both efficacy outcomes and comprehensive cytotoxicity analyses, clearly oriented toward translatable research with potential positive clinical impact.

I recommend this manuscript for publication in Nature Communications. However, I do have several comments/ questions that, if addressed, would further strengthen the work.

1. Can authors offer any commentary on why they chose docosanoic acid as their lipophilic conjugate and their rationale as a whole regarding choosing their selected conjugates?

2. The authors relay that the docosanoic conjugate is the most lipophilic conjugate tested. While looking at chemical structure one could come to the same conclusion, it would be nice to see an inclusion of some metric of lipophilicity of the selected conjugates showing this range, perhaps logP studies or some other analysis.

3. Docosanoic acid has been implicated in anti-inflammatory processes in the body. The authors show that their DCA conjugated siRNA has an improved cytotoxic profile compared to other conjugates. Could the authors comment or provide any data regarding if the anti-inflammatory of DCA may be implicated in this study? Would its inclusion further increase skin retention due to its lipophilicity and some method of anti-inflammatory processes?

Version 1:

Reviewer comments:

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Reviewer #2

(Remarks to the Author)
The authors have sufficiently addressed my comments, provided clarification where needed, and modified the main text accordingly. Overall, the study demonstrates compelling pre-clinical data, and presents a clear path for clinical trials to investigate their lead siRNA conjugate. I recommend the manuscript for publication in Nature Communications.

Reviewer #3

(Remarks to the Author)
Authors have effectively answered and responded to comments made by myself and other reviewers. This manuscript is even stronger. I recommended this manuscript for acceptance.

Very minor detail: on line 95 " 95 For that, We assessed the cellular uptake of cyanine-" should be..."For that, we assessed the cellular uptake of cyanine-" lowercase we. Again very minor.

Remarks to the author

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This is a nicely conceived study demonstrating that intradermally administered siRNAS can provide proprolonged silencing of the Jak1gene which has downstream effects on inflammtory cytokines and chemokines. Overall this study has important implications for treating dermal inflammatory conditions like psoraiasis and eczema. The only shortcoming is that data feom scrambled or other control siRNAS seem to be missing.

We thank the reviewer for pointing out the absence of a non-targeting control (NTC) siRNA in the pig studies as a limitation. While we acknowledge this limitation, we wish to emphasize the data gathered from the human tissue samples in Figure 1. These samples showed clear confirmation that NTC and PBS treatments are indistinguishable in their effect on JAK1 mRNA levels, which supports the specificity and on-target action of the therapeutic JAK1 siRNA.

In the minipig studies, three controls were included in parallel:

- The solvent to control for procedural effects.
- The human siRNA compound (non-cross-reactive) to control for chemistry-related effects, effectively serving as an NTC.
- The minipig-specific compound to assess pharmacodynamic-related effects.

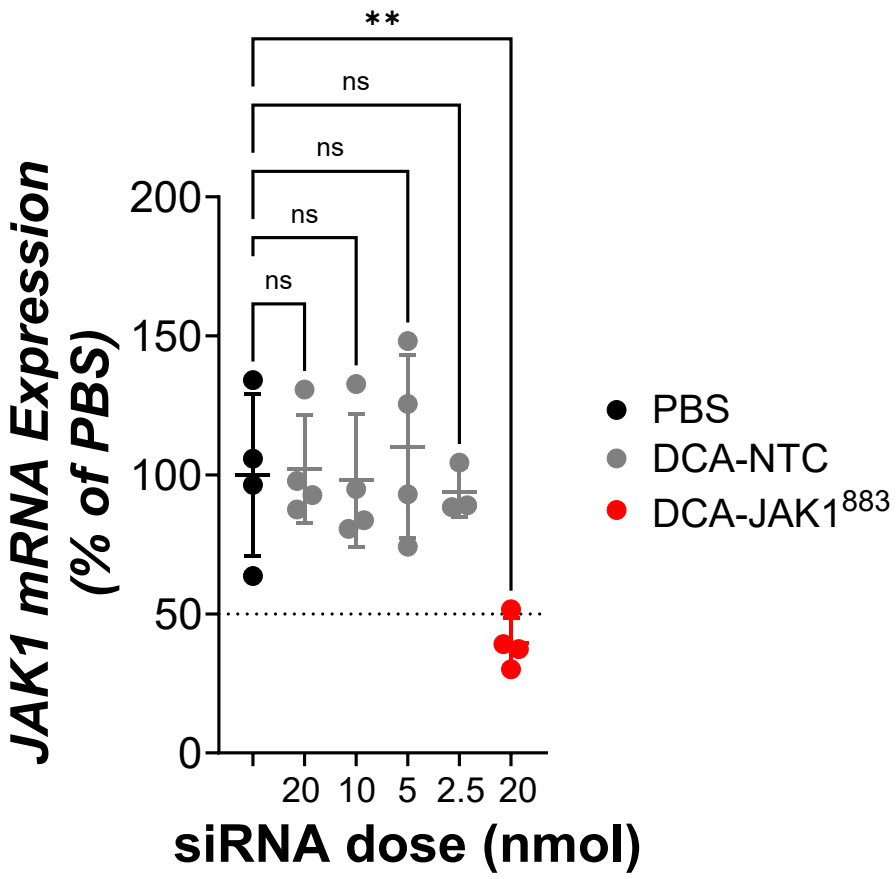
We selected the solvent as an additional control rather than a dedicated NTC for three reasons:

1. The human compound provided a functional NTC in a non-cross-reactive species.
2. This approach aligns with the 3Rs principles (Replacement, Reduction, Refinement) for large-animal studies.
3. It reflects the clinical trial setting, where patients receive placebo (solvent) rather than an NTC siRNA.

This design allowed differentiation of adverse events related to the procedure/solvent from those linked to siRNA chemistry or sequence. This design and data was formally accepted by the FDA as part of the IND-enabling package supporting Phase I/II trial initiation.

We did conduct however an *ex vivo* pig study, similar to the human *ex vivo* study, where pig skin was injected with PBS, DCA-NTC and DCA-JAK1^{883} and showed no significant difference in expression between NTC and PBS. The figure has been added to the supplemental information (Supplementary Figure 6), as well as the paragraph below

discussing the limitation of the pig study, in the discussion section:



“Another limitation is the absence of a non-targeting control (NTC) siRNA group in the pig study. Due to the significant cost constraints of large animal studies, and the inclusion of these data into the Investigational New Drug (IND)-enabling toxicity package, PBS (the placebo used in clinical trial), was selected as the control. However, the impact of the siRNA chemistry in the form of an NTC control is mitigated by several lines of evidence: human biopsy data showed that NTC-treated and PBS-treated tissues were identical in their inability to affect JAK1 mRNA levels; additionally, the human-targeting JAK1^{3033} compound—which acts as an NTC in pigs since it is not cross-reactive—demonstrated no alterations in CBC, blood chemistry, or inflammatory markers at various timepoints and in two different conjugates. These findings support the on-target specificity of the JAK1 siRNA and confirm that the siRNA chemistry itself does not cause non-specific inflammation”

Reviewer #2

The manuscript presents a lipophilic siRNA conjugate strategy for treating skin diseases. The authors provide favorable safety and PK/ PD profile for administering siRNA in a localized manner without significant systemic drug exposure. The data presented across large animal model and clinically relevant human skin tissue is very encouraging. The overall study is well-planned and executed and presented in a clear and systematic manner, however, there are certain aspects of the work which need additional experimentation and clarification:

1. Why did D-JAK13033 display considerable variability in the epidermal layer? Provide more explanation. Would increasing the sample size resolve the statistical significance?

We thank the reviewer for their comment. There are two potential explanations for variability in the epidermal layer: 1) the albumin-binding, amphiphilic nature of the dendrimer conjugate that can leak out of the epidermal layer into the solution more readily, decreasing its retention and efficacy; and 2) the variability coming from the separation process of epidermal layer, as it is a very thin layer being peeled.

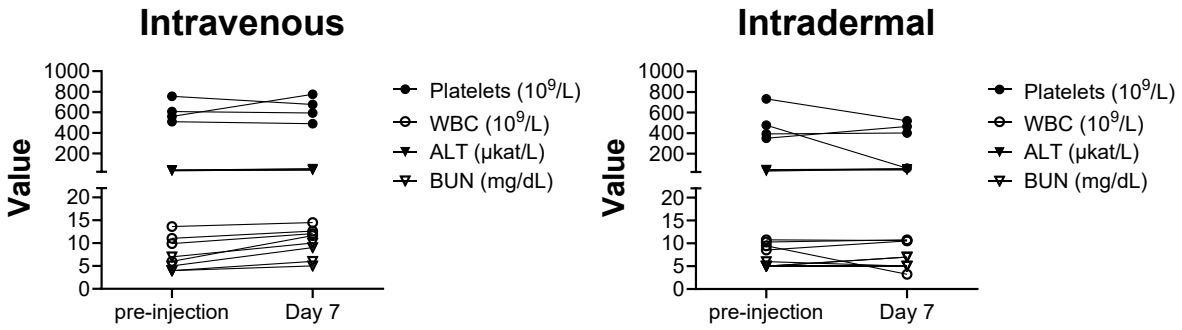
While the statistics show no significant difference in the epidermis for dendrimer ($p=0.07$) which might be improved with a larger sample size, it ultimately does not affect the conclusion of the stronger potency observed in the dermis and epidermis with DCA-siRNA which is the lead chemistry. We have adjusted the main text to discuss this, and reproduced below:

"However, D-JAK1^{3033} while showing a silencing trend, it had considerable variability in the epidermal layer, failing to achieve statistical significance ($p=0.07$). This increased variability in the epidermis following an intradermal injection could be a result of the chemistry-dependent distribution away from the site of injection, leakage into the media, or issues with the separation of the various skin layers for processing."

2. It is recommended to include non-targeting (scrambled) siRNA control in the in vivo porcine experiment to showcase differences in inflammatory markers. Since the siRNA is targeting a protein involved in an inflammatory cascade, there could be some unforeseen changes in the baseline levels which may not be accurately captured from untreated and PBS-treated study groups. This is evident from supplemental Figure S2, where PBS-treated human biopsy samples appear to have lower JAK1 mRNA levels compared to the non-targeting control group.

We thank the reviewer for their critical feedback. We have elaborated on this general limitation in our response to Reviewer 1 (above) and addressed it within the manuscript discussion. Particularly for the impact on inflammatory markers, no changes were observed at any of the 17 measured inflammatory markers (Figure S4) from pigs injected with D-JAK1^{3033} and DCA-JAK^{3033} and no alteration to pathology readouts from WBC and blood chemistry counts (Figure 2C). Here the siRNAs are JAK1^{3033} which

target only the human transcript and not the pig, essentially acting as NTC controls in pigs. We have also added WBC and blood chemistry data from the JAK1^{883} PK and efficacy study (Figure 3) which also show no changes to the levels pre-dosing in either intravenous or intradermal dosing of DCA-JAK1^{833} siRNAs. The panel is reproduced below, and added to supplemental figure S5 – E):



Specifically concerning Figure S2, we confirm there is no statistically significant difference between the NTC and PBS human biopsy groups in either Figure 1D or the revised Figure S2 (where statistics against both controls have now been added). The slight, non-significant trend observed is limited to the epidermal layer, which we believe is a result of normal experimental variation. Since this variation is not replicated in Figure 1D and did not reach statistical significance in any comparison, we are confident it does not impact our overall conclusions. Lastly, in our in vivo comparisons to untreated and PBS, which in Figure S2 trend to have slightly lower JAK1 expression than the NTC, this leads to a more conservative measure of apparent potency of the siRNA.

3. What does the significance between PBS and on-target JAK1 siRNA look like in Figure S2?

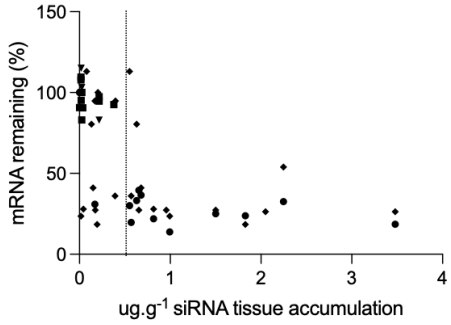
We have added the significance to PBS to Figure S2, where slight statistic changes were observed to the epidermal layer, but no changes were observed in the dermal layer which is the site of injection.

4. The authors report more than 8 weeks of siRNA retention in porcine tissue after intradermal injection. However, for the efficacy studies, they selected 1 month as the end-point. Why was this timeline chosen? Additionally, is there a siRNA threshold concentration needed at the target site to achieve therapeutic benefit?

We appreciate the reviewer's comment regarding the duration of the efficacy study. While we do have supporting siRNA retention data in skin up to 8 weeks (Figure 2B), this specific time point was limited in sample size (single biopsy at 8 weeks compared to n=2-3 at 4 weeks) and utilized the more potent JAK1^{3033} siRNA sequence rather than

the pig-reactive JAK1^{883} sequence used in the efficacy study. Given these constraints, and the high cost associated with large animal studies, we chose the 4-week endpoint to ensure robust knockdown. The ongoing clinical investigation aims to assess clinical efficacy 4 months after the last injection day.

By combining many of our experiments where pharmacokinetic (PK) and pharmacodynamic (PD) assessments were performed with different concentrations of siRNA and different application modes (transepidermal, topical, or intradermal), we were able to estimate the minimum required level for silencing. In this approach, we only included biopsies from time points where samples were collected at least 7 days after the last application or injection to ensure that silencing had time to establish and that non-functional, recently applied siRNA does not blur the PK accumulation without pharmacodynamic activity. it allowed us to estimate that to achieve 50% silencing, a threshold of 0.5 µg/g of JAK1^{883} is required in the dermis. However, many limitations exist to this approach (among them, for example: two different biopsies are used for PK and PD; different experiments or pigs are combined in the same graph; different modes of application, different timepoints from last application (7 to 28 days) etc.), which is why we did not include this into a publication.



5. Make sure the graph axis is consistent across different figure panels for better visualization. For instance, in Fig. 3b, y-axis for CXCL levels in dermis vs. epidermis has different axis points.

Figure 4B y-axis have been adjusted and are now consistent

6. Given the translational potential of this technology and the mention of an on-going Phase 2a human clinical trial, the authors should add a discussion about other delivery systems (transdermal patches, ointments, and so on) that may be utilized as an alternative to painful intradermal injections where the intended treatment surface area is larger.

We have now added a discussion on alternative delivery strategies beyond intradermal injections, reproduced below:

“Beyond intradermal injections, there are currently multiple advanced delivery systems being explored to administer therapeutics, to the skin.[35] These non-invasive strategies can potentially serve as alternatives for treating diseases that require coverage of much larger skin surface areas. Key examples include microneedle patches (MNs) [36,37], which create transient microchannels for enhanced drug uptake, ointments and other topical formulations, and novel delivery enhancers utilizing materials such as ionic liquids[38] and star particles [39]. These strategies can potentially be utilized as alternatives to deliver therapeutic siRNAs to much larger skin surface areas for diseases that require bigger coverage.”

Reviewer #3

This manuscript leverages a less commonly studied porcine model alongside a widely used ex vivo model, providing a valuable comparative perspective. The authors adeptly integrate lipid chemistry and RNA chemistry to enhance gene silencing efficacy and tissue retention in the skin, which is the body's largest organ. Furthermore, the analysis of unconjugated, dendrimer-conjugated, and their docosanoic siRNA conjugates allow for meaningful comparison and context within the field. Notably, the authors report increased silencing in JAK1 for their leading siRNA conjugate in both human skin and the porcine model. This work makes important advances in intradermal delivery technology. The inclusion of comparative data across different siRNA conjugates, presented in a more human-compatible porcine model, provides novel insight and may inform future research directions. In addition, the manuscript addresses a central challenge in the field: extrahepatic delivery. Building on previous research, the authors progress this area toward greater clinical relevance, as evidenced by the ongoing Phase 2a human clinical trial of the leading compound, DCA-JAK1. The manuscript is compelling in its broad inclusion of both efficacy outcomes and comprehensive cytotoxicity analyses, clearly oriented toward translatable research with potential positive clinical impact. I recommend this manuscript for publication in Nature Communications. However, I do have several comments/ questions that, if addressed, would further strengthen the work.

We thank the reviewer for their comments and feedback

1. Can authors offer any commentary on why they chose docosanoic acid as their lipophilic conjugate and their rationale as a whole regarding choosing their selected conjugates?

We thank the reviewer for their comment. DCA was chosen based on our previous work where we compared a much larger range of hydrophobic conjugates (Biscans et al *Nucleic Acid Research 2019*) and DCA showed the highest retention in the skin, which was then further confirmed to provide the strongest siRNA efficacy in skin (Tang et al

Molecular Therapy 2022). Similarly, the recently developed Dendrimer conjugate (Fakih et al *Molecular Therapy Nucleic Acid 2023)* showed promising skin retention and activity, while having an amphiphilic character and lower hydrophobicity than DCA, which warranted us to compare it to DCA in human skin tissue and larger animal models in the current study. We have elaborated the results section discussing the conjugate rationale to capture these reasons:

"From the library of conjugates previously assessed, docosanoic acid (DCA) demonstrated the highest skin retention and siRNA activity in mouse skin tissue, making it a compelling candidate for further evaluation.[18,25] Additionally, a recently developed amphiphilic dendrimer conjugate achieved effective skin delivery while being significantly less hydrophobic than DCA, warranting a direct comparison between the two.[19]"

2. The authors relay that the docosanoic conjugate is the most lipophilic conjugate tested. While looking at chemical structure one could come to the same conclusion, it would be nice to see an inclusion of some metric of lipophilicity of the selected conjugates showing this range, perhaps logP studies or some other analysis.

We thank the reviewer for the excellent suggestion. We have previously compared the hydrophobicity of the conjugates used in the form of retention time on reverse phase HPLC analysis, which clearly shows the hydrophobic nature of the tested compounds and providing a nice range of hydrophobicity tested. That work was published (Fakih et al *Molecule Therapy Nucleic Acids 2023*) and the figure (Figure 1A bottom) has been reproduced below. We have also edited the main text to highlight this as mentioned in response to the first comment.

[Figure Redacted]

3. Docosanoic acid has been implicated in anti-inflammatory processes in the body. The authors show that their DCA conjugated siRNA has an improved cytotoxic profile compared to other conjugates. Could the authors comment or provide any data regarding if the anti-inflammatory of DCA may be implicated in this study? Would its inclusion further increase skin retention due to its lipophilicity and some method of anti-inflammatory processes?

We thank the reviewer for this comment. While Docosahexaenoic Acid (DHA), the poly-unsaturated version of Docosanoic acid (DCA), plays an important role in anti-inflammatory processes, we did not find any evidence of DCA's role in inflammatory processes. This is due to DHA being a polyunsaturated fatty acid (22 carbon single bonds, 6 double bonds) which are precursors of biologically active lipid mediators. However, DCA is fully saturated (22 carbon single bonds,0 double bonds) lipid and does not play an anti-inflammatory role. Therefore, we do not believe that the DCA conjugate in DCA-siRNA is playing an anti-inflammatory role, an assumption that is further supported by our DCA conjugated non-targeting control which shows similar JAK1 expression to PBS (Figure 1D).

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

This is an important study demonstrating that dermal delivry of ASOs targeting the Jak0Stat pathway can be used to ammeliorate inflammatory skin conditions. The concerns of the previous reviews have been adequately addressed.

We thank the reviewer for their feedback and we are glad we were able to address the concerns.

Reviewer #2 (Remarks to the Author):

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Very minor detail: on line 95 " 95 For that, We assessed the cellular uptake of cyanine-" should be..."For that, we assessed the cellular uptake of cyanine-" lowercase we. Again very minor.

We have fixed this typo, we thank the reviewer for their keen eye.