Nonselective Chemical Inhibition of Sec7 Domain-containing ARF GTPase Exchange Factors

Kiril Mishev, K., et al.

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REPORT: (The report shows the major requests for revision and author responses. Minor comments for revision and miscellaneous correspondence are not included. The original format may not be reflected in this compilation, but the reviewer comments and author responses are not edited, except to correct minor typographical or spelling errors that could be a source of ambiguity.)

TPC2018-00145-RA 1st Editorial decision – *accept with minor revisions* March 22, 2018

In addition to addressing the points raised by the reviewers, it will be important to make your ms more accessible to a non-specialist reader and to highlight the relevance and potential of identifying a new ARF-GEF inhibitor.

RESPONSE: We rewrote the Discussion with an emphasis on expanding the inhibitor toolbox to study the ARF GEF protein functions in plant cells. We outlined more clearly the added value of the use of Secdin, as revealed by the impact on both BFA-sensitive and BFA-resistant ARF GEFs and the unique intracellular phenotypes that can be induced by Secdin alone or in combination with BFA. We also provided an example for the usefulness of Secdin when the functions of the plasma membrane proteins are explored, of which the ARF GEF-dependent endocytic trafficking cannot be manipulated with BFA. In the Results section, we introduced more comprehensive explanations that deal with the combined chemical treatment assays carried out to address the effect of Secdin on the plant endomembrane system. Additionally, we tried to address all Editorial remarks concerning the style of figures and supplemental materials, including font sizes, suggested graph and table adjustments as well as additional explanations on the sampling methods. We also converted two supplemental figures (former SI Fig. 4 and SI Fig. 9) to main figures (Figures 4 and 8 from the revised version) to meet the criteria of the Journal for supplemental material content. All changes introduced in the revised version of the manuscript are highlighted.

---------- Reviewer comments:

[Reviewer comments shown below along with author responses]

TPC2018-00145-RAR1 1st Revision received June 25, 2018

Reviewer comments and **author responses**:

Reviewer #2:

The manuscript by Mishev et al. presents a novel chemical compound, termed Secdin, which inhibits or modulates several instances of endomembrane transport in plants and binds ARF-GEFs, thus probably interfering with ARF-dependent trafficking events. After the initial description of how Secdin was isolated, the authors lay out a very comprehensive and detailed study of its effects, where they see a number of inhibitory effects on post-Golgi trafficking. Importantly, they are wary of potential unspecific side-effects and perform appropriate tests. The authors assess a number of post-Golgi trafficking events and found that all of them are inhibited to some degree, the

strongest effects being MVB/LE to vacuole traffic. The authors convincingly demonstrate that ARF-GEFs are targets of Secdin, and that, interestingly, Secdin presumably does not affect ARF GDP-GTP conversion, suggesting a different mode of action. Also, it seems that apart from changes in MVB/LE structure, all effects are rather transitory and more of a delaying or slowing down characteristic.

The manuscript is generally well structured; experiments have been carried out carefully and described in high detail. The authors go a long way in elucidating the molecular mechanism of action by identifying ARF-GEFs as targets and demonstrating that the mode of action differs from that of other, known ARF-GEF inhibitors. Of course, further experiments are required to get to a working model, but this would clearly be outside of the scope of this already data-rich manuscript. For example, whether ARF-GEF recruitment is altered under Secdin treatment, perform DARTs with truncated/mutated ARF-GEFs, experiments in non-plant organisms etc. I have only a couple of points:

Point 1. In the fluorometric assays for ARF1 activation, it would be good to include a series with a known inhibitor, or any other kind of positive control.

RESPONSE: In our nucleotide exchange kinetics assays, we use human protein constructs for which potent inhibition by at least one chemical compound has been published recently (Benabdi et al., 2017, Biochemistry 56, 5125-5133). We made sure that the protein samples had GEF activity in the same range as those measured previously, as can readily be assessed from the raw data. We believe that we provide the relevant control instead of repeating the inhibition assays with other compounds. In the Results section, we introduced an explanatory sentence referring to our already published results with known ARF GEF inhibitors and human GEF protein constructs that had later been probed with Secdin as well. Additionally, in the revised manuscript, we included results with a newly purified Arabidopsis BIG5^{Sec7} protein construct. We found that it was highly active toward ARF1 in vitro, with a nucleotide exchange efficiency similar to that of human BIG1^{Sec7} under equivalent conditions (k_{obs}= 0.017 s^{-1} for BIG1^{Sec7}; k_{obs}=0.013 s^{-1} for BIG5^{Sec7}), and that it was strongly inhibited by BFA used as a positive control treatment (Figure 8A and Supplemental Figure 7; see also the updated Methods section).

Point 2. ARNO or Brag2 should be included in the DARTs assay.

RESPONSE: As suggested by the Reviewer, we carried out DARTS assays with the small human ARF GEF, cytohesin-2/ARNO. For these experiments, we used human cell cultures and detected the endogenous ARNO with an anti-ARNO antibody. As expected from the GEF activity assay, Secdin failed to bind ARNO in DARTS (see Supplemental Figure 6D).

Point 3. The rationale for excluding BIG2 in DARTS assay should be given.

RESPONSE: For our DARTS analysis with Secdin and BFA, we used antibodies recognizing the native GNOM and BIG5 and generated HA-tagged protein overexpression constructs with the Gateway system to detect BIG1, BIG3, BIG4, GNL1, and GNL2 levels. We also attempted to generate an HA-tagged BIG2 construct with Gateway vectors, but we could not obtain a correct expression clone. Alternatively, we obtained the recently published pBIG2-BIG2-GFP construct (Kitakura et al., 2017, Plant Cell Physiol. 58, 1801-1811), which we used to transform Arabidopsis PSB-D cell suspension cultures. However, the BIG2-GFP protein levels seemed to be too low to monitor the BIG2 proteolytic digestion in the presence of the studied compounds. Therefore, the DARTS assay was not reliable. A reason for the absence of BIG2 in our DARTS analysis was also included into the Results section of the revised manuscript. Accordingly, throughout the manuscript, we modified the statements for Secdin interaction with all Arabidopsis ARF GEFs, which now read as Secdin interaction with all tested Arabidopsis ARF GEFs.

Point 4. The authors present a suite of rather complex experiments in which different pharmacological agents are combined with Secdin. This part is very difficult to follow for non-experts and would benefit from a better description of the rationale behind each experiment.

RESPONSE: We fully agree with the Reviewer's comment and modified the Results section accordingly. In particular, we explained in more detail the reason for the Secdin pretreatment of BRI1-GFP crosses with endomembrane markers prior to incubation with specific inhibitors of TGN/EE or MVB/LE. The experimental design, including concomitant incubations with Secdin and BFA, was also clarified as part of our research on the impact of Secdin on vesicle trafficking processes assisted by BFA-sensitive ARF GEFs.

Point 5. The Discussion would also benefit from a rewrite and a clear sub-structure. As it is now, it is again very difficult to follow for non-experts. Maybe the authors could also discuss possible use-cases for this drug as a tool for novel discoveries.

RESPONSE: To comply with the Reviewer's remark, we rewrote the Discussion and introduced subsections. As outlined above (see our response to the Editor's suggestions), in the new version of the Discussion, we stressed the need for expanding the inhibitor toolbox to study the ARF GEF protein functions in plant cells. We stated more clearly the added value of Secdin, as revealed by the impact on both BFA-sensitive and BFA-resistant ARF GEFs and the unique intracellular phenotypes that can be induced by Secdin alone or in combination with BFA. We also provided an example for the usefulness of Secdin when the functions of plasma membrane proteins are explored of which the ARF GEF-dependent endocytic trafficking cannot be modified with BFA.

Point 6. If Secdin can indeed bind the catalytic site of the Sec7 domain, it is very strange that this does not inhibit ARF conversion; this should be also addressed in the Discussion.

RESPONSE: The Reviewer is right for raising such a concern. We needed to clarify that our DARTS experiments with Secdin and the full-length Arabidopsis ARF GEFs imply that Secdin binds all tested ARF GEFs. However, the GEF activity assay was carried out with only the Sec7 domains of the human BIG1, ARNO, and BRAG2. Secdin failed to inhibit the activity of the Sec7 domains (see Figure 8B and Supplemental Figure 7), suggesting that Secdin does not bind this domain in the examined human ARF GEFs (small, ARNO and BRAG2; large, BIG1). In addition, the Sec7 domain of the Arabidopsis BIG5 was expressed. GEF activity assays were carried as for the human ARF GEFs and showed that, whereas BFA inhibited the Sec7 BIG5 activity, Secdin had no statistically significant effect (see Figure 8A and Supplemental Figure 7). Altogether, these data support the conclusion that the inhibitory effect of Secdin is not mediated by direct binding and inhibition of the Sec7 domain. As our experiments did not confirm the docking predictions that Secdin binds the Sec7 domain, we removed the latter from the manuscript. DARTS showed that Secdin does not bind the full-length small ARF GEF ARNO (see Supplemental Figure 6D) that correlated with the lack of ARNO inhibition (Figure 8B and Supplemental Figure 7). In contrast, the large Arabidopsis ARF GEFs seemed to be susceptible to Secdin (Figures 7B and 7C); hence, we cannot exclude the possibility that Secdin targets domains other than Sec7 in this protein family. However, biochemically, it is very challenging to produce full-length large ARF GEFs with preserved activity that can be used for confirmatory binding experiments and ARF1 activation assays with Secdin in vitro. This possible mode of action has been clarified in the Discussion of the revised manuscript.

Reviewer #3:

The manuscript by Mishev et al., describes the identification of Secdin through a high-throughput screen and the depth characterization of its inhibitory effects. The trafficking inhibitor causes multiple phenotypes, including delayed secretion and endocytosis, impaired protein recycling and enhanced vacuolar degradation. Interestingly, the compound in combination with ARF GEF inhibitor Brefeldin A induces the formation of hybrid endomembrane structures. Application of a protease protection assay known as DARTS suggested that Secdin binds to all Arabidopsis ARF GEFS, however fluorescence kinetics shows that it does not interfere directly with the activity of representative human Sec7 domains. Although the mechanism of action of Secdin is not yet resolved, the study presents an elegant characterization of a new trafficking inhibitor that can be useful in studying ARF GEF protein functions in plants.

Points In favor: In depth and elegant characterization of Secdin, which has unique characteristics compared to other trafficking inhibitors. The study used interdisciplinary approaches to characterize the compound's inhibitory activity. Secdin broadens the pharmacological toolbox to dissect ARF GEF protein functions in plants.

Points detracting: DARTS is a useful approach to demonstrate potential interaction with a compound, but confirmatory studies are required to demonstrate binding. The authors examine the effect of Secdin on the activity of representative human Sec7 domains, resulting in no inhibition. However, the manuscript does not address the effect of Secdin in non-plant systems.

Point 1. Given that binding of Secdin to all ARF GEFs is stated in the abstract, confirming the binding of at least selected ARF-GEFs is deemed necessary. Alternatively, the main focus and conclusions of the manuscript should be modified.

RESPONSE: (see also our response to Reviewer #2): We agree with the Reviewer that, besides DARTS, additional in vitro binding studies are needed to prove that Secdin binds to the ARF GEFs. However, in contrast to small ARF GEFs, like ARNO, that can be produced as full-length functional proteins, the characterization of the BIG and GBF family of large ARF GEFs in human has been hindered by the difficulty in purifying protein constructs encoding full-length functional gene products (Richardson et al., 2012, Dev. Cell 22, 799-810). Nevertheless, we produced the Sec7 domain of the Arabidopsis BIG5 in bacteria. This protein was able to activate the human ARF1 in vitro. However, we failed to carry out differential scanning fluorimetry (DSF) experiments, because the BIG5 Sec7 domain was unstable at low temperatures, even in the absence of the compound. Later GEF activity assays showed that Secdin had no statistically significant inhibitory effect on the Sec7 domain of BIG5 (see Figure 8A and Supplemental Figure 7), supporting the conclusion that most probably Secdin does not bind the Sec7 domain of BIG5. Similarly, Secdin failed to inhibit and bind full-length functional ARNO (see Figure 8B and Supplemental Figures 6D and 7). To comply with the Reviewer's suggestion, we have modified the conclusions.

Point 2. The effect of Secdin in non-plant systems should be examined.

RESPONSE: To address this comment, we collaborated with Wim Annaert's laboratory (VIB KU Leuven) to test the effect of Secdin on the Golgi morphology in HeLa cells by immunofluorescence. The obtained results are summarized in Supplemental Figure 8 in the revised manuscript. Secdin dispersed the Golgi marker from the perinuclear region into the cytoplasm similarly to cells treated with BFA and AMF-26 (Ohashi et al. 2012. J Biol Chem 287:3885-3897; Zeeh et al. 2006. J Biol Chem 281:11805-11814; Supplemental Figure 8).

TPC2018-00145-RAR1 2nd Editorial decision – *acceptance pending* **July 2, 2018**

We are pleased to inform you that your paper entitled "Nonselective chemical inhibition of Sec7 domain-containing ARF GEFs in Arabidopsis" has been accepted for publication in The Plant Cell, pending a final minor editorial review by journal staff. At this stage, your manuscript will be evaluated by a Science Editor with respect to scientific content presentation, compliance with journal policies, and presentation for a broad readership.

Final acceptance from Science Editor **July 17, 2018**