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The structure-activity relationship of the salicylimide derived inhibitors of UDP-sugar producing pyrophosphorylases

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ABSTRACT
UDP-sugars are key precursors for biomass production in nature (synthesis of cellulose, hemicellulose, etc.). They are produced de novo by distinct UDP-sugar producing pyrophosphorylases. Studies on the roles of these enzymes using genetic knockouts were hampered by sterility of the mutants and by functional-complementation from related enzyme(s), hindering clear interpretation of the results. In an attempt to overcome these difficulties, we turned to the reverse chemical genetics approaches to identify compounds which interfere with the activity of those enzymes in vivo. Hit expansion on one of such compounds, a salicylimide derivative, allowed us to identify several inhibitors with a range of activities. The present study provides a structure-activity relationship for these compounds.

Genetic studies on mechanisms of UDP-sugar formation were frequently hampered both by reproductive impairment of the resulting mutants. and by functional-complementation from related enzyme(s). It was thus difficult to unequivocally assess the in vivo roles of the separate UDP-sugar forming enzymes. We have recently shown that several compounds, identified by screening a small molecule chemical library, served as potent UDP-glucose pyrophosphorylase (UGPase) and UDP-sugar pyrophosphorylase (USPase) inhibitors, and that some of them were also effective in in vivo studies. The identified inhibitors were inhibiting both UGPase and USPase activities, possibly reflecting common structural features at or near active sites of these enzymes. Dose-response studies revealed that the identified inhibitors had IC50 values in the μM-range against each of the target enzymes.

The potential of our identified candidate compounds for plant uptake was evaluated first by analyzing their physicochemical properties (quantitative estimation of plant translocation, QEPT). This analysis suggested that cmpd #6, a salicylimide derivative, was by far the most promising. The in vivo effects of the inhibitors were evaluated in two plant systems: Arabidopsis pollen germination, and Arabidopsis cell culture. The cmpd #6 acted as strong inhibitor of both pollen germination tests and cell growth, which verified the results of our QEPT analysis, and indicated that indeed the inhibitor was able to enter plant cells to reach its target(s). In order to identify even stronger inhibitors of UGPase and/or USPase, we performed a so-called hit-expansion, where compounds which are derived from the top-candidate(s) are analyzed to identify superior inhibitors. We used cmpd #6 as starting point, since it was relatively effective in vitro (effect in μM-range against both UGPase and USPase), in vivo (effect in μM-range against both pollen germination and cell culture), had acceptable QEPT and there were many commercially available analogs.

Two rounds of hit-expansion allowed us to evaluate the importance of different parts of the cmpd #6 molecule, and gave clues to its structure-activity-relationship (SAR) for our target enzymes (Figure 1). The analyses have revealed that the salicylimide derivative (aromatic ring A and R1-R3) was present in most active compounds (inhibition of both UGPase and USPase), with the exception of cmpd #6H which may represent a different mode of inhibition. Modifications of the first portion of the linker (R3, between ring A and B) led to abolished inhibition, whereas changes of the length of the linker were acceptable (R4). Considering aromatic ring B, modifications of the meta-positions (R5 and R7) led to inactive inhibitors, whereas changes in the para-position (R6) could alter the strength of the inhibitors, and halogens were accepted, while O-methyl (cmpd #6D) and S-methyl (cmpd #6D2) substituents led to increased potency. Little/no inhibition was observed for dimethylamine substituents in the para-position (R6) against USPase activity, whereas UGPase was inhibited which could mean that this compound (cmpd #6B) may be used as a starting point to identify more selective inhibitors. Compound #6D2, which was the most potent inhibitor (Figure 1), contains two parts which resemble known metabolites: ring A and substituents R1-R3 resemble salicylic acid (SA), while ring B and substituents R3-R7 are similar to p-coumaric acid. These two compounds were, however, not able to cause similar effects as cmpd #6D and #6D2, neither in vivo nor in vitro. Both cmpd #6D and #6D2 also contain structures which could potentially serve as metal-
chelators (R3) and/or Michael-acceptors (R4), but those were unlikely to play a role for UGPase/USPase inhibition, as they were also present in inactive compounds.

Plants are known to synthesize different forms of glycosylated SA, the major being SA-2-β-D-glucoside (SAG) and the minor SA-Glc ester, with both forms being inactive and stored in the plant vacuoles. In analogy, perhaps a #6D2-glucoside can also be formed (by glycosylation of ring A and R1-R2) and subsequently sequestered. Transgenic plants with altered UGPase gene expression were shown to have altered levels of SAGs. If such sequestering occurs, however, it should also happen with cmpd #6A and #6K, which were inactive against the target enzymes, but still blocked pollen germination.

Based on the SAR originating from the lead compound (e6), the most promising candidate inhibitors, based on their potency, were cmpd #6D and #6D2, and they were subsequently selected for further studies. They acted as uncompetitive inhibitors against both UGPase and USPase (with e.g. inhibition constants for cmpd #6D2 of 30 and 40 µM against UTP and Glc-1-P, respectively, for UGPase; and 70 and 50 µM against UTP and Glc-1-P, respectively, for USPase), and were strongly inhibiting during pollen test, with their apparent inhibition constants of less than 1 µM.

The uncompetitive mode of inhibition by cmpd #6D and #6D2 seems useful for in vivo studies, as the effect of the inhibitor will not be outcompeted (as in the case of competitive inhibitors) by accumulating substrates. In a more recent study, activity of Arabidopsis UDP-N-acetyl glucosamine pyrophosphorylase2 (UAGPase2) was also found to be inhibited by cmpd #6D, further underscoring common structural features at or near the active sites of distinct pyrophosphorylases, which differ in substrate specificity.

Overall, the results suggest that cmpd #6D and #6D2 and their analogs may represent useful tools to study in vivo roles of the UDP-sugar metabolizing pyrophosphorylases and that their use may serve as a complement to the genetic approaches. Further hit expansion on cmpd #6 analogs and, perhaps, on other inhibitors identified during the chemical library screening, may yield compounds which will act even stronger against the pyrophosphorylases than cmpd #6D2 (cmpd #6H and #6I may be promising starting-points as

<table>
<thead>
<tr>
<th>Inhibitor in-house name</th>
<th>Effect</th>
<th>Inhibition (%) of UGPase at 50 µM</th>
<th>Structure of compound</th>
</tr>
</thead>
<tbody>
<tr>
<td>#6D2</td>
<td>79</td>
<td>yes</td>
<td>-H -O-(CO)-(NH)-(CO)- -(CH)=(CH)- yes -H -S-Me -H</td>
</tr>
<tr>
<td>#6H</td>
<td>56</td>
<td>yes ***</td>
<td>-O- -(CO)-(NH)-(CO)- -Me - - - -</td>
</tr>
<tr>
<td>#6D</td>
<td>54</td>
<td>yes</td>
<td>-H -O-(CO)-(NH)-(CO)- -(CH)=(CH)- yes -H -O-Me -H</td>
</tr>
<tr>
<td>#6B</td>
<td>54</td>
<td>yes</td>
<td>-H -O-(CO)-(NH)-(CO)- -(CH)=(CH)- yes -H -O-Me -H</td>
</tr>
<tr>
<td>#6D4</td>
<td>51</td>
<td>yes</td>
<td>-H -O-(CO)-(NH)-(CO)- -(CH)=(CH)- yes -H -O-Me -H</td>
</tr>
<tr>
<td>#6I</td>
<td>47</td>
<td>yes</td>
<td>-H -O-(CO)-(NH)-(CO)- -(CH)=(CH)- yes -H -O-Me -H</td>
</tr>
<tr>
<td>#6</td>
<td>32</td>
<td>yes</td>
<td>-H -O-(CO)-(NH)-(CO)- -(CH)=(CH)- yes -H -Cl -H</td>
</tr>
<tr>
<td>#6D1</td>
<td>N.S.</td>
<td>yes *(NH2)- (CO)- (NH)-(CO)- (CH)=(CH)- yes -H -H -H</td>
<td></td>
</tr>
<tr>
<td>#6K</td>
<td>N.S.</td>
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<td></td>
</tr>
<tr>
<td>#6A</td>
<td>N.S.</td>
<td>yes *(NH2)- (CO)- (NH)-(CO)- (CH)=(CH)- yes -H -H -H</td>
<td></td>
</tr>
<tr>
<td>#6E</td>
<td>N.S.</td>
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<tr>
<td>#6C</td>
<td>N.S.</td>
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<tr>
<td>#6F</td>
<td>N.S.</td>
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<td></td>
</tr>
<tr>
<td>#6D3</td>
<td>N.S.</td>
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<td></td>
</tr>
<tr>
<td>PA</td>
<td>N.S.</td>
<td>yes *(NH2)- (CO)- (NH)-(CO)- (CH)=(CH)- yes -H -H -H</td>
<td></td>
</tr>
</tbody>
</table>

Figure 1. In vitro structure-activity-relationship for UGPase/USPase inhibitors. Regions in which modifications were tolerated included mainly R4 and R6. N.S. indicates no significant effect according to Students T-test, p < 0.05, N = 2, * = 1,3-benzoxazole-2(3H)-thione, ** = 1-(4-bromophenyl)ethanone, *** = 1-(3,4-dichlorophenyl)ethanone.
they may function differently from the other cmpd #6 ana-
logs), and could be useful for in vivo studies. Finding inhibi-
tors that selectively affect a given pyrophosphorylase activity
should be one of the priorities.\textsuperscript{16,17}

\textbf{Disclosure of Potential Conflicts of Interest}

No potential conflicts of interest were disclosed

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