Note

MurA as a Primary Target of Tulipalin B and 6-Tuliposide B

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Received August 29, 2013; Accepted September 26, 2013; Online Publication, December 7, 2013
[doi:10.1271/bbb.130663]

(--)-Tulipalin B and (+)-6-tuliposide B were confirmed to inhibit MurA in vitro. However, contrary to fosfomycin, these compounds showed potent inhibitory activities against MurA overexpressing Escherichia coli, especially in the presence of UDP-GlcNAc. These observations suggest that these compounds induced bacterial cell death not through a MurA malfunction, but in such a MurA-mediated indirect manner as the inhibition of other Mur enzymes.

Key words: tuliposide; tulipalin; MurA

Tuliposides are secondary metabolites occurring mainly in the Liliaceae and Alstroemeriaeae.¹⁻³ Tuliposides A, B, D, E, and F have been reported together with their chemical structures.⁴⁻⁷ All tuliposides are composed of a d-glucose moiety and mono- or dihydroxylated methylenebutanoyl side chain(s). These side chains can be released by either pH-dependent or enzymatic lactonization to afford tulipalin A (2-methylenebutyrolactone) or (2-C13)-butyrolactone. Tulipalins are known as causative agents of contact dermatitis, called “tulip fingers.”⁸ We have recently revealed the antiulcerative activity of this class of compounds against tulip pathogenic fungus, together with their respective pigment-inducing or -inhibiting activities against Gibberella zeae and Fusarium oxysporum f. sp. tulipae.⁹ We have also reported the antibacterial activities of (--)tulipalin B and (+)-6-tuliposide B.¹⁰ The broad structure-activity relationship (SAR) in that study demonstrated (--)tulipalin B to be the principal agent for antibacterial activity, suggesting that (+)-6-tuliposide B could be the natural precursor in the acidic organelle of tulip anthers to be converted to (--)tulipalin B after a microbial attack.¹⁰ Based on this SAR, we speculated that the primary target of tulipalin B as an antibacterial agent could be cytoplasmic MurA, like the related compound, cnicin,¹¹ isolated from Cnicus benedictus L. MurA is the bacterial dual-substrate enzyme that catalyzes the coupling of phosphoenolpyruvate (PEP) and UDP-N-acetylglucosamine (UDP-GlcNAc) to afford enolpyruvylated UDP-GlcNAc that is a primitive building unit of peptidoglycan.¹² The biosynthesis of peptidoglycan initiated by MurA is a specific and critical survival system for bacteria. The discovery and development of new inhibitors targeting MurA have therefore gained prominence.¹³ To validate our hypothesis, we evaluated the inhibitory potency of (--)tulipalin B and (+)-6-tuliposide B against MurA by in vitro experiments and in bacterial cells.

To establish the inhibitory actions of (--)tulipalin B and (+)-6-tuliposide B against MurA, we first evaluated the direct interactions between MurA and tuliposides or tulipalins. Partially purified MurA from Escherichia coli and Enterobacter cloacae was incubated for 10 min with 0.1 mM of synthetic (--)tulipalin B, (+)-tulipalin B, (+)-6-tuliposide B, (+)-3'-epi-6-tuliposide B¹⁰ and commercially available tulipalin A in the presence or absence of 1.0 mM UDP-GlcNAc. It is well known that the recruitment of PEP by MurA occurs only after a UDP-GlcNAc intake. Therefore, a UDP-GlcNAc supplement is often used to detect the specificity of PEP mimics against MurA.¹⁴ After preincubation, the remaining relative activities of MurA were evaluated by measuring the amount of inorganic free phosphate by the malachite green method.¹³ Table 1 shows the results. The inhibitory activities of tuliposides and tulipalins against MurA showed a similar trend to the previously reported antibacterial activities.¹⁰ Both (3'R)- and (3'S)-6-tuliposide Bs or (--) and (+)-tulipalin Bs showed almost the same inhibitory activities, while structurally similar tulipalin A showed much less activity than tulipalin Bs and 6-tuliposide Bs. The remaining activities of MurA were dramatically increased.

Table 1. Remaining MurA Activities (%) against Tuliposides and Tulipalins

<table>
<thead>
<tr>
<th>Compound</th>
<th>MurA (E. coli) with UDP-GlcNAc (1.0 mM)</th>
<th>MurA (E. coli) without UDP-GlcNAc</th>
</tr>
</thead>
<tbody>
<tr>
<td>(+)-6-Tuliposide B</td>
<td>25</td>
<td>35</td>
</tr>
<tr>
<td>epi(+)-6-Tuliposide B</td>
<td>17</td>
<td>49</td>
</tr>
<tr>
<td>(--)Tulipalin B</td>
<td>2.8</td>
<td>1.7</td>
</tr>
<tr>
<td>(+)-Tulipalin B</td>
<td>2.5</td>
<td>2.9</td>
</tr>
<tr>
<td>Tulipalin A</td>
<td>87</td>
<td>54</td>
</tr>
</tbody>
</table>

*Enzymes were preincubated with compounds for 10 min. †0.1 mM UDP-GlcNAc was used.

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Abbreviations: SAR, structure-activity relationship; MurA, UDP-GlcNAc enolpyruvyltransferase; UDP-GlcNAc, UDP-N-acetylglucosamine; UDP, uridine diphosphate
ed under conditions without UDP-GlcNAc ((+)-6-tuliposide B, 25% to 91%; (-)-tulipalin B, 2.8% to 52%). These results proved the inhibitory effects of (-)-tulipalin B and (+)-6-tuliposide B to be enhanced by UDP-GlcNAc against MurA at the enzyme level. 10) Tulipalins and tuliposides would form the adducts with UDP-GlcNAc by the catalytic action of MurA, as did cnicin as shown in Fig. 1.

We next conducted a growth-recovery experiment by using MurA-overexpressing the Escherichia coli BL21 strain to confirm the antibacterial target of (-)-tulipalin B and (+)-6-tuliposide B. The Escherichia coli murA gene was transformed into the E. coli BL21 strain by using plasmid pET-41a(+) according to the conventional protocol. The overexpression of MurA inducted with IPTG (50 µM) was confirmed by 10% SDS–PAGE, showing the 44.7-kDa band corresponding to native MurA (Fig. S1; See Biosci. Biotechnol. Biochem. Web site). A control vector (mock) expressing or MurA-overexpressing E. coli BL21 strain was treated with (-)-tulipalin B, (+)-6-tuliposide B and fosfomycin, which is a known specific MurA inhibitor. 15) Antibacterial assays were performed according to a previously described method 10) in the presence of 25 µg/mL of kanamycin and 50 µM of IPTG. Figure 2A shows that the growth of mock-transformed E. coli was nearly completely inhibited by 0.1 mM of fosfomycin, while MurA-overexpressing E. coli showed remarkable growth at the same concentration (Fig. 2A). These results well reflect that the overexpression of MurA compensated for the decreased activity of the endogenous enzyme. Contrary to our expectation, the growth of E. coli overexpressing MurA was rather suppressed by the (-)-tulipalin B treatment. A tetracycline or norfloxacin treatment did not affect the growth of MurA-overexpressing E. coli due to their different respective targets, the 30S ribosomal subunit and DNA gyrase (Fig. 2D). The addition of UDP-GlcNAc significantly enhanced the antibacterial effects of (-)-tulipalin B and (+)-6-tuliposide B against E. coli overexpressing MurA in a dose-dependent manner (Fig. 2B and C). The effect of UDP-GlcNAc was not apparent in E. coli overexpressing MurA treated with fosfomycin (Fig. 2A–C), and E. coli overexpressing MurA treated with tetracycline or norfloxacin showed completely the same growth, even in the presence of 1.0 mM UDP-GlcNAc (Fig. S2). The present growth-recovery experiment proves the involvement of both MurA overexpression and a high concentration of UDP-GlcNAc in the growth inhibition by (-)-tulipalin B or (+)-6-tuliposide B. MurA is known to catalyze the unusual binding of the butanoate side-chain of cnicin, which is an equivalent of
tulipalin B, to UDP-GlcNAc. This UDP-GlcNAc–cnicin adduct has been considered to trigger the malfunction of MurA and to induce cell death. However, the actual fate of this adduct is still unclear. The binding of cnicin, (−)-tulipalin B or (+)-6-tuliposide B to MurA is possibly slow and reversible because they did not form a covalent bond to the enzyme, like fosfomycin. The high amount of MurA and UDP-GlcNAc can promote the production of a UDP-GlcNAc–tulipalin B adduct which may then inhibit the function of such subsequent enzymes as MurB and MurC in the presence of overexpressed MurA. The MurA-related initial cell wall synthesis is finely regulated by various factors, for instance the concentration of UDP-GlcNAc, inorganic phosphate, or UDP-N-acetylmuramic acid (UDP-MurNAc), the product of MurB. It might therefore be possible for a faultily formed adduct to interrupt subsequent pathway too. Further kinetic investigations would be needed to clarify the detailed relationship between MurA inhibition and bacterial cell death by these inhibitors.

In conclusion, we have proved that both (−)-tulipalin B and (+)-6-tuliposide B inhibited bacterial MurA in vitro as we hypothesized. However, differently from fosfomycin, a high concentration of UDP-GlcNAc significantly enhanced the inhibitory effects of (−)-tulipalin B and (+)-6-tuliposide B against E. coli overexpressing MurA. (+)-6-Tuliposide B seemed to be a unique phytoanticipin, and its less potent antibacterial activity was enough to protect pollen from bacterial attack, because a rich amount of (+)-6-tuliposide B is specifically accumulated in the anthers of tulip. Further investigation of the UDP-GlcNAc–tulipalin B adduct might lead to the development of a new type of antibacterial agent.

Acknowledgment

This work was supported in part by grant-aid for scientific research (A) provided by the Japan Society for the Promotion of Science of Japan.

References