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Differential antibacterial properties of the MurA inhibitors terreic acid and fosfomycin

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Abstract

Terreic acid is a metabolite with antibiotic properties produced by the fungus *Aspergillus terreus*, but its cellular target remains unknown. We recently reported that terreic acid inactivates the bacterial cell wall biosynthetic enzyme MurA *in vitro* by covalent reaction with residue Cys115 in a similar manner as the MurA-specific antibiotic fosfomycin. To address if terreic acid also targets MurA *in vivo*, we conducted antibacterial studies using selected *E. coli* strains in parallel with fosfomycin. While overexpression of MurA conferred resistance to fosfomycin, it did not protect cells treated with terreic acid. Furthermore, flow cytometry revealed that the antibiotic action of terreic acid appears to be primarily bacteriostatic, as opposed to the bactericidal action observed for fosfomycin. Combined, the data suggest that MurA is not the molecular target of terreic acid and that the antibiotic activity of terreic acid proceeds through a different mechanism of action. The methodology applied here provides a reliable and convenient tool to rapidly assess the potential of newly discovered *in vitro* inhibitors to target residue Cys115 of MurA in the cell.

Keywords

Antibiotics; Drug discovery; Flow cytometry; Natural products; Terreic acid

INTRODUCTION

The enzyme UDP-N-acetylglucosamine enolpyruvyl transferase (MurA, EC 2.5.1.7) is critical for the survival of most bacteria [1]. MurA catalyzes the first step in biosynthesis of the bacterial cell wall, and inactivation of this enzyme by the antibiotic fosfomycin (1, Fig. 1) results in bacterial cell lysis and death [2]. Therefore, MurA has become a validated and attractive target for the design of novel antibiotic agents [3, 4]. Several *in vitro* inhibitors of MurA have been reported in the past decade [5, 6], including natural products such as the sesquiterpene lactone cnicin [7, 8] and tuliposides [9, 10]. However, none of these inhibitors have been shown to target MurA in the cell. Overexpression of wild type MurA has been shown to render cells tolerant to fosfomycin treatment, presenting a convenient method for characterization of other MurA inhibitors in bacterial cell cultures [11]. Moreover, a

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CONFLICT OF INTEREST

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naturally occurring mutation, Cys115Asp, renders MurA resistant to fosfomycin and other Cys115-modifying inhibitors while retaining full catalytic activity [12–14].

We recently reported that the natural product terreic acid (**2**, Fig. 1), produced by the fungus *Aspergillus terreus*, is a covalent in vitro inhibitor of MurA from *Escherichia coli* as well as from *Enterobacter cloacae* [13]. The antibiotic properties of terreic acid were first described more than 60 years ago [15], but its molecular target(s) in bacteria remain unknown. Chemically, terreic acid is a quinone epoxide that covalently attacks the MurA Cys115 residue in a similar manner to fosfomycin [13, 16]. The potent in vitro inhibition of MurA by terreic acid suggested that this compound might exert its antibacterial activity through specific targeting of MurA in the cell. To test this hypothesis, we employed a combination of bacterial growth and flow cytometry studies using selected *E. coli* strains, both with and without overexpression of wild type MurA and the fosfomycin-resistant Cys115Asp mutant. However, terreic acid was not able to induce a significant level of cell lysis as compared to fosfomycin, and overexpression of wild type or Cys115Asp MurA did not protect the cells from terreic acid. These results suggest that MurA is not the molecular target of terreic acid, and that the antibiotic activity of terreic acid instead proceeds through a different mechanism of action. The methodology applied here provides a reliable and convenient tool to rapidly assess the potential of newly discovered in vitro inhibitors to target Cys115 of MurA in the cell.

MATERIALS AND METHODS

Materials

Chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. Terreic acid was obtained from Tocris Bioscience (Ellisville, MO). Cloning and overexpression of *E. cloacae* wild type MurA and the Cys115Asp mutant was performed as previously described [17]. Overexpression of *E. cloacae* MurA (both wild type and Cys115Asp) was carried out in *E. coli* BL21(DE3) cells (Agilent Technologies, Santa Clara, CA).

Antibacterial studies

Bacterial cell density was assessed by absorbance measurements at 600 nm (OD_{600}) using a SpectraMax 340PC plate reader from Molecular Devices (Sunnyvale, CA). Three sample sets of *E. coli* BL21(DE3) cells were grown in LB broth with appropriate antibiotics at 37°C: one control set with no MurA overexpression, one with overexpression of wild type MurA, and one with overexpression of Cys115Asp MurA. Cells were grown until $OD_{600}=0.5$, then were treated with 0.6 mM IPTG to induce protein expression. After 30 min, cells were treated with serial dilutions of fosfomycin or terreic acid, ranging from 0–1 mM. All cell cultures were allowed to grow for an additional 4 h before determining final cell density. Bacterial IC_{50} values were determined by fitting data to Equation 1 using relative OD (expressed as the ratio of treated over untreated cells). Experiments were repeated independently three times.

$$OD = OD_{\min} + \frac{OD_{\max} - OD_{\min}}{1 + \left(\frac{[I]}{IC_{50}}\right)^n} \quad \text{Equation (1)}$$

Flow cytometry

BL21(DE3) cells were inoculated in 50 mL LB broth to $OD_{600}=0.1$ in both the presence and absence of fosfomycin or terreic acid (16 μM or 130 μM , respectively; concentrations are slightly above the bacterial IC_{50} concentrations for these drugs). The cultures were grown until untreated control cells reached an OD_{600} of 0.9–1.0. Cell suspensions were prepared and stained using the LIVE/DEAD® BacLight bacterial viability kit (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions with minor variations as indicated. The experiment was independently repeated twice. Cell suspensions were stained with SYTO®9 (5 μM final concentration) and propidium iodide (PI; 2.5 μM final concentration) immediately before measuring. SYTO®9 and PI are nucleic acid-binding fluorescent dyes which stain bacterial cells depending on membrane integrity, and are suitable to differentiate between live and dead cells. SYTO®9 is cell-permeable and stains all bacteria, while PI is cell-impermeable and only stains cells with compromised membranes. Samples were measured on a FACSCalibur flow cytometer (BD BioSciences, San Jose, CA). Excitation was carried out at 488 nm for both SYTO®9 and PI. SYTO®9 excitation was detected in FL1 with a 530/30 nm bandpass filter, while PI excitation was detected in FL3 with a 670 nm longpass filter. Data was analyzed using Flowjo analysis software (Treestar Inc., Ashland, OR). The entire population was gated on side scatter (SSC) versus forward scatter (FSC) to eliminate noise. Four gates were set for analysis of the fluorescent stains: cells with a high stain intensity for SYTO®9 and a low intensity for PI (lower right quadrant of plot, Gate 1) were assumed to have intact cell membranes, while cells exhibiting low stain intensity for SYTO®9 and high for PI (upper left quadrant of plot, Gate 3) were considered to have compromised cell membranes, i.e. pre-lysed cells. The cell population staining with medium intensity for both SYTO®9 and PI (Gate 2) was considered to be in the transition between these two stages [18], while the subset with low intensity stain for both SYTO®9 and PI were considered cell debris, i.e. completely lysed cells (Gate 4).

RESULTS AND DISCUSSION

The use of overexpression strains in the identification of cellular targets was recently reported [11]. We therefore devised a combination of existing methods, modified to suit our needs and based on our own previous studies of MurA, to determine if in vitro MurA inhibitors such as terreic acid are able to exert antibiotic activity in bacterial cultures by inducing cell lysis [2]. Additionally, this method enables determination of whether the antibacterial activity of a compound is dependent on the presence of the MurA Cys115 residue that is targeted by epoxide moieties present in both fosfomycin and terreic acid [13, 19].

Effect of terreic acid on bacterial growth

We first compared the cellular activity of terreic acid in bacterial cell cultures to the activity exerted by fosfomycin. This was accomplished by measuring dose-response curves of bacterial cultures upon treatment with serial dilutions of the two inhibitors, using an untreated culture as a control. Bacterial IC_{50} values were determined for cells with genomically expressed MurA, plasmid-induced overexpression of wild type MurA, or plasmid-induced overexpression of Cys115Asp MurA (Fig. 2). As expected, fosfomycin acted as a potent antibiotic against cells that express genomic MurA (bacterial $IC_{50} = 4 \mu M$). Overexpression of wild type MurA protected the cells from fosfomycin, although a decrease of approximately 25 % in cell density was repeatedly observed at inhibitor concentrations above 20 μM . The Cys115Asp mutation rendered cells completely resistant to fosfomycin at concentrations as high as 500 μM (Fig. 1, Table 1). In contrast, we did not observe a response upon treatment with terreic acid comparable to the response upon fosfomycin treatment in any of the three cell strains. Additionally, overexpression of MurA did not appear to protect the cells, with bacterial IC_{50} values ranging from 47–92 μM (Fig. 2, Table 1).

Notably, cell density in the presence of terreic acid reached a growth plateau at approximately 40 % of the density observed for untreated cells. Fosfomycin treatment, by comparison, reduced the cell density to nearly zero, indicative of complete cell lysis. The residual cell density at high terreic acid concentrations led us to investigate whether the cells were still viable by pelleting cells treated with high concentrations of terreic acid and resuspending them in fresh media. The cells were able to resume growth at levels comparable to untreated cells (data not shown). These observations suggest that terreic acid inhibits cell growth independent of MurA without inducing cell lysis. The observed differences in cellular effects by fosfomycin and terreic acid may also be attributed in part to the potentially lower solubility of quinone-based structures such as terreic acid which could, in turn, adversely affect cell permeability.

Effect of terreic acid on bacterial cell membrane integrity

We employed flow cytometry to further evaluate the ability of terreic acid to induce cell lysis by enabling visualization of cell membrane integrity following treatment with inhibitors. Cultures treated with terreic acid contained very low quantities of cells with compromised membranes as compared to cultures treated with fosfomycin (Fig. 3): 130 μM terreic acid yielded 75.5 % uncompromised cells and 20.8 % compromised cells, with the remaining 3.7 % scattered outside the gates. Similar distribution was observed for the untreated controls, which suggests that terreic acid halts cell growth without compromising cell membrane integrity. Conversely, the bacteriolytic activity of 16.5 μM fosfomycin was clearly evident upon treatment, resulting in 5.5% uncompromised cells and 84.6 % compromised cells, with the remaining 9.9 % scattered outside the gates. These observations support the findings of the bacterial IC_{50} studies in that terreic acid does not appear to induce cell lysis but rather exhibits bacteriostatic activity.

CONCLUDING REMARKS

We recently reported that the natural product terreic acid is a potent inhibitor of MurA in vitro, covalently interacting with residue Cys115 [13]. Since fosfomycin exerts antibiotic activity through covalent modification of the same residue in MurA, we evaluated whether MurA is the cellular target of terreic acid by bacterial growth studies, including flow cytometry. However, terreic acid merely halted cell growth without inducing significant cell lysis, and overexpression of MurA did not protect the cells. Combined, these data indicate that MurA is not the primary target of terreic acid in the cell and suggest that the antibiotic activity of terreic acid proceeds through a different mechanism of action in which it may have multiple cellular targets with a cumulative antibacterial effect.

The combination of existing methods employed in this study are ideally suited to rapidly and reliably assess the potential of newly discovered in vitro inhibitors to target MurA in the cell, providing a comprehensive approach in the process of identifying promising lead compounds for drug discovery. Inhibitors that exert antibiotic activity by selectively targeting Cys115 in MurA must fulfill two criteria: induction of lysis in cells harboring genomic wild type MurA, and inactivity against cells overexpressing wild type or Cys115Asp mutant enzyme. Although a potent in vitro inhibitor of MurA, we conclude that terreic acid is a poor candidate for the development of MurA-specific drugs. In addition to terreic acid, two other classes of natural products, cnicins and tuliposides, have recently been demonstrated to inactivate MurA in vitro through covalent modification of Cys115. It was concluded that the antibiotic activity of these compounds primarily results from inhibition of MurA [7, 9, 10]. However, the present study clearly demonstrates the divergence between in vitro potency and MurA selectivity; thus, it remains to be seen whether cnicins and tuliposides indeed target MurA in the cell.

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Abbreviations used

MurA	UDP-N-acetylglucosamine enolpyruvyl transferase
IPTG	isopropyl- β -D-thiogalactopyranoside
SSC	side scatter
FSC	forward scatter

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Fig. 1.
Structures of MurA inhibitors fosfomycin (1) and terreic acid (2).

Fig. 2.

Overexpression of MurA does not protect *E. coli* from terreic acid. Dose-response curves were determined for terreic acid treatment of BL21(DE3) cells with no MurA overexpression (▲), MurA wild type overexpression (■), and MurA Cys115Asp overexpression (●). Parallel experiments were conducted for fosfomycin treatment of cells with no MurA overexpression (△), MurA wild type overexpression (□), and MurA Cys115Asp overexpression (○). Data were fit to Equation 1, yielding the bacterial IC_{50} values listed in Table 1.

Fig. 3.

Terreic acid does not induce significant cell lysis in *E. coli*. Flow cytometry heat maps of *E. coli* BL21(DE3) cells untreated (A), treated with 16.5 μ M fosfomycin (B), or treated with 130 μ M terreic acid (C) indicate that terreic acid does not compromise cell membrane integrity as compared to treatment with fosfomycin. Gates (outlined) are defined as follows: (1) cells with uncompromised cell membranes; (2) intermediate cell population; (3) cells with compromised cell membranes; (4) cell debris. The number of cell counts in each gate is listed as a percentage of the total. Cells in gates 2–4 are considered to have compromised membranes.

Table 1Bacterial IC₅₀ values for terreic acid and fosfomycin

Overexpression	Bacterial IC ₅₀ (μM)	
	Terreic Acid	Fosfomycin
None	92±1	4±0.1
MurA wild type	70±2	>20
MurA Cys115Asp	47±1	>500