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Improved production of cytotoxic thailanstatins A and D through metabolic engineering of *Burkholderia thailandensis* MSMB43 and pilot scale fermentation

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ABSTRACT

Thailanstatin A (TST-A) is a potent antiproliferative natural product discovered by our group from *Burkholderia thailandensis* MSMB43 through a genome-guided approach. The limited supply of TST-A, due to its low titer in bacterial fermentation, modest stability and very low recovery rate during purification, has hindered the investigations of TST-A as an anticancer drug candidate. Here we report the significant yield improvement of TST-A and its direct precursor, thailanstatin D (TST-D), through metabolic engineering of the thailanstatin biosynthetic pathway in MSMB43. Deletion of *tstP*, which encodes a dioxygenase involved in converting TST-A to downstream products including FR901464 (FR), resulted in 58% increase of the TST-A titer to 144.7 ± 2.3 mg/L and 132% increase of the TST-D titer to 14.6 ± 0.5 mg/L in the fermentation broth, respectively. Deletion of *tstR*, which encodes a cytochrome P450 involved in converting TST-D to TST-A, resulted in more than 7-fold increase of the TST-D titer to 53.2 ± 12.1 mg/L in the fermentation broth. An execution of 90 L pilot-scale fed-batch fermentation of the *tstP* deletion mutant in a 120-L fermentor led to the preparation of 714 mg of TST-A with greater than 98.5% purity. The half-life of TST-D in a phosphate buffer was found to be at least 202 h, significantly longer than that of TST-A or FR, suggesting superior stability. However, the IC_{50} values of TST-D against representative human cancer cell lines were determined to be greater than those of TST-A, indicating weaker antiproliferative activity. This work enabled us to prepare sufficient quantities of TST-A and TST-D for our ongoing translational research.

1. Introduction

Cancer has the second highest mortality rate only next to cardiovascular diseases and is usually associated with specific genetic and epigenetic changes in cellular processes.[1–3] Pre-mRNA splicing inhibitors, including FR901464 (FR; Fig. 1),[4–6] spliceostatins,[7,8] thailanstatins,[9] pladienolides,[10] FD-895,[11] and herboxidiene (GEX1),[12–14] are a promising class of bacterial natural products or their derivatives for cancer drug discovery and development. FR demonstrated not only potent antiproliferative activity against an array of human cancer cell lines with IC_{50} values in the low nanomolar (nM) range but also the ability to prolong the life of tumor-bearing mice.[5] However, due to its instability as well as unacceptable levels of toxicity, FR has been abandoned from a drug development program.[15] Mechanistically FR and spliceostatin A (SSA) inhibit pre-mRNA splicing by binding to SF3b, a subcomplex of the U2 small nuclear ribonucleoprotein in the spliceosome.[16]

FR was discovered by Nakajima et al. from *Pseudomonas* sp. No. 2663 (recently re-classified as *Burkholderia* sp. FERM BP-3421[17]) through cell-based screenings;[4] thailanstatin A (TST-A; Fig. 1) was discovered by us from *Burkholderia thailandensis* MSMB43 through genome mining.[9] TST-A biosynthesis in MSMB43 and FR biosynthesis in FERM BP-3421 appear to use the same biosynthetic logic,[9,17,18] raising the possibility that these two strains are either identical or very closely related. Three oxygenase activities, including a flavin-dependent monooxygenase (FMO) domain encoded by *fr9H*/*tstGH*, a cytochrome P450 encoded by *fr9R*/*tstR* and a Fe(II)/α-ketoglutarate-dependent dioxygenase encoded by *fr9P*/*tstP*, were proposed in the formation of a heavily decorated tetrahydropyran ring (Fig. 1). Specifically, the Fr9P/TstP dioxygenase was shown to convert a C1 acetic acid group in TST-A into a hemiketal group in FR through oxidative

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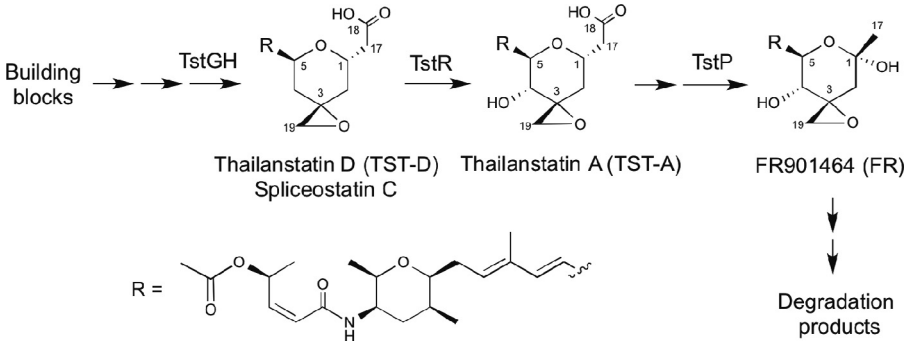

Fig. 1. Structures and biosynthetic relationship of TST-D, TST-A and FR901464.

decarboxylation. As such, TST-A is a precursor of FR. The carboxylic acid moiety in TST-A is beneficial for drug development for the following reasons. First, this carboxylic acid moiety enables TST-A to be more stable than the hemiketal-containing FR.[9] Second, when the carboxylic acid group of TST-A is modified to an ester linkage, which is more lipophilic and enhances the cell membrane permeability of the derived compound, the cytotoxicity of the compound is significantly increased.[8]

Our investigation of TST-A as an anticancer drug candidate encountered a shortage of compound supply, primarily due to its low titer in bacterial fermentation, modest stability and a very low recovery rate during purification.[9] Here we report the significant yield improvement of TST-A through metabolic engineering of the thailanstatin biosynthetic pathway in MSMB43 and pilot scale fermentation. During the course of this research, a direct TST-A precursor was isolated and named as thailanstatin D in 2013 (TST-D; Fig. 1), which turned out to be identical to the recently reported spliceostatin C (SSC).[17] The antiproliferative activity and stability of TST-D were assessed and reported as well.

2. Materials and methods

2.1. Strain, plasmid and media

All strains and plasmids used in this study are listed in Table 1. Luria-Bertani (LB) agar or medium was used with appropriate concentration of antibiotics for routine cultivation of *B. thailandensis* MSMB43 strains or *Escherichia coli* strains. A 2S4G medium[17] was used for bacterial fermentation in flasks; a slightly modified 2S4G medium composed of 40 g/L glycerol, 12.5 g/L HySoy soypeptone, 2 g/L $(NH_4)_2SO_4$, 0.1 g/L $MgSO_4{\cdot}7H_2O$ and 2 g/L $CaCO_3$ (pH 7.0) was used for fed-batch bacterial fermentation in a fermentor. The concentrated feed medium contained 400 g/L glycerol, 20 g/L $(NH_4)_2SO_4$ and 1 g/L $MgSO_4{\cdot}7H_2O$ (pH 7.0).

2.2. Construction of gene deletion mutants of B. thailandensis MSMB43

A multiplex PCR method[20] was used to create gene deletion mutant strains of MSMB43 (Fig. S1), as we routinely did in previous works.[19,22] PCR primers used for mutagenesis and for verification of mutant genotypes are listed in Table S1.

2.3. Bacterial fermentation, extraction and chromatographic purification of TST-A and TST-D

Bacterial fermentation, extraction, isolation and purification were performed according to schemes and associated description given in the Supplementary material (Figs. S2 and S3). TST-D was identified to be identical to spliceostatin C (SSC)[17] with extensive UV, IR, HR-MS and NMR analyses (Figs. S4–S15).

2.4. Titer determination by LC–MS analysis

The titers of TST-A and TST-D in fermentation broth were quantified with an Agilent 6400 Series Triple Quadrupole LC–MS system equipped with an Agilent Eclipse Plus C18 column (φ 4.6 × 100 mm, 3.5 μm) and a UV detector. Briefly, each 0.5 mL of fermentation broth was sampled at various time points and was extracted twice with equal volume of ethyl acetate. Two extracts were combined, dried in a refrigerated CentriVap centrifugal vacuum concentrator (Labconco) and subsequently re-suspended in 0.5 mL of acetonitrile and filtered through a 0.22 μm filter. Two microliters of such acetonitrile solution was injected into the LC–MS system. The LC solvents included buffer A (water with 0.1% formic acid, FA) and buffer B (acetonitrile with 0.1% FA). The column was eluted with a linear gradient from 15% to 55% buffer B in 35 min, monitored at 235 nm and with a flow rate of 0.5 mL/min. MS signals were collected in positive mode under the following conditions: N_2 gas temperature, 325 °C; gas flow, 10 L/min; nebulizer pressure, 20 psi;

Table 1
Strains and plasmids used in this study.

Strains and plasmids	Relevant genotype or description	Source or reference
Strains		
E. coli DH5α	General *Escherichia coli* host strain for DNA cloning	Lab stock
E. coli S17-1	*E. coli* donor strain for interspecies conjugation	Lab stock
*Bth*WT	*Burkholderia thailandensis* MSMB43 wild-type strain	CDC
BthΔtstP::FRT	*B. thailandensis ΔtstP::FRT* intermediate insertion mutant	This study
BthΔtstP	*B. thailandensis ΔtstP* final marker-free deletion mutant	This study
BthΔtstR::FRT	*B. thailandensis ΔtstR::FRT* intermediate insertion mutant	This study
BthΔtstR	*B. thailandensis ΔtstR* final marker-free deletion mutant	This study
Plasmids		
pBS854-Tp	*Tp*^{r}; donor of a trimethoprim-resistance cassette	[19]
pEX18Tc	*Tc*^{r} *oriT*$^+$ *sacB*$^+$; gene replacement vector with MCS	[20]
pBMTL3-Flp2	Flp endonuclease expression vector	[21]
pEX18Tc-*ΔtstP::Tp*	*Tc*^{r} *Tp*^{r} *oriT*$^+$ *sacB*$^+$; *tstP* gene replacement construct	This study
pEX18Tc-*ΔtstR::Tp*	*Tc*^{r} *Tp*^{r} *oriT*$^+$ *sacB*$^+$; *tstR* gene replacement construct	This study

CDC, US Centers for Disease Control and Prevention; *Tp*^{r}, trimethoprim resistant; *Tc*^{r}, tetracycline resistant.