Enzyme Inhibitors Cause Multiple Effects on Accumulation of Monoterpene Indole Alkaloids in Catharanthus Roseus Cambial Meristematic Cell Cultures

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ABSTRACT

Background: Enzyme inhibitors have been used for the clarification of biosynthesis of natural products. Catharanthus roseus cambial meristematic cell (CMC) culture has been established and proved to be a better monoterpenoid indole alkaloid (MIA) producer than C. roseus dedifferentiated cell (DDC) culture. However, little is known about the inter-relationship of the MIA-biosynthetic genes with respect to their transcription.

Objective: To clarify effects of alteration of one gene transcription on transcript levels of another genes in MIA-biosynthetic pathway, and how the accumulation of MIAs in CMCs are influenced by the alteration of their biosynthetic gene transcript levels.

Materials and Methods: 3-Hydroxy-3-methylglutaryl-CoA reductase (HMGR) inhibitor Lovastatin and 1-deoxy-D-xylulose 5-phosphate synthase (DXS) inhibitor Clomazone were fed to C. roseus CMC cultures. The contents of MIAs were qualified by High Performance Liquid Chromatography and the transcript levels of the relevant genes were measured by qRT-PCR.

Results: Lovastatin improved the accumulation of MIAs via increasing the transcription of their biosynthetic genes encoding DXS1, tryptophan decarboxylase (TDC), loganic acid methyltransferase (LAMT), strictosidine synthase (STR), desacetoxyvindoline-4-hydroxylase (D4H) and ORCA3 (a jasmonate-responsive transcriptional regulator), whereas clomazone reduced the contents of MIAs and the mRNA levels of the corresponding genes.

Conclusion: The biosynthesis of MIAs in C. roseus is manipulated via a complex mechanism, the knowledge of which paves the way for rationally tuning metabolic flux to improve MIA production in C. roseus CMCs.

Key words: Cambial meristematic cell, Catharanthus roseus, enzyme inhibitor, monoterpenoid indole alkaloids,

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INTRODUCTION

More than 3000 different monoterpenoid indole alkaloids (MIAs) are found in eight plant families (e.g., Apocynaceae, Loganiaceae and Rubiaceae), some of which have been reported to possess powerful biological and pharmacological activities. In Catharanthus roseus, over 100 different MIAs have been characterized, including ajmalicine with anti-arrhythmic and antihypertensive activities, and vinblastine and vincristine used as anticancer medicines. Due to their high-value pharmacological activities, many efforts have been made to study the biosynthesis of MIAs.

MIA biosynthetic pathway in C. roseus is complex and usually illustrated in four stages: (I) monoterpene biosynthesis, including the production of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), and the formation of monoterpene geraniol derived from IPP and DMAPP; (II) iridoid biosynthesis, i.e., the conversion of geraniol to iridoid glycoside secologalnin; (III) early MIA biosynthesis, i.e., the production of strictosidine aglycone via the coupling of secologalnin and tryptamine derived from tryptophan, and consequent deglycosylation; (IV) late MIA biosynthesis, including synthesis of all the monoindole alkaloids (e.g., vindoline, catharantine and ajmalicine) derived from strictosidine aglycone, and bisindole alkaloids (e.g., vinblastine and vincristine) produced from coupling between vindoline and catharantine.

In plants, the biosynthesis of IPP occurs via two metabolic pathways: the mevalonic acid (MVA) pathway and the mevalonolactone 4-phosphate (MEP) pathway. Characterization of which pathway provides IPP for biosynthesis of MIAs would pave the way for refining metabolic flux to enhance yields of MIAs in plants and in culturable plant cells/tissues. Different strategies, including inhibitor experiments, incorporation of labeled precursors and analyses of transgenic lines and mutants were employed to elucidate the metabolic source of isoprenoid units, and some progresses were made. However, all these efforts are mostly focused on early MIA-biosynthesis steps, such as relationships between MVA pathway and MEP pathway or between isoprenoid IPP and DMAPP flux and production of iridoid intermediates. Inhibitors of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) and 1-deoxy-D-xylulose 5-phosphate synthase (DXS) involved in MVA and MEP pathway, respectively, have been used as additional tools to study regulation of isoprenoid production in plants. Herein, we used HMGR inhibitor Lovastatin and DXS inhibitor Clomazone to alter the production of IPP and DMAPP derived from either MVA or MEP and also investigated their effects on downstream MIA-biosynthetic steps. Our previous work has established a C. roseus cambial meristematic cell (CMC) culture system, which is a better MIA producer than both C. roseus dedifferentiated cell (DDC) cultures and hairy root.
cultures. In this article, we investigated growth characteristics, yields of MIAs (ajmalicine, vindoline and catharanthine) and transcription of key MIA-biosynthetic genes in *C. roseus* CMCs treated with lovastatin and clomazone, respectively. These findings may provide basis for rationally tuning metabolic flux to enhance production of MIAs in *C. roseus* CMCs.

**MATERIALS AND METHODS**

**Chemicals**

Vindoline, catharanthine, ajmalicine, lovastatin, clomazone (2-[2-chlorophenyl]-4, 4-dimethyl-3-isoxazolidinone) and ammonium acetate were obtained from Aladdin (Aladdin Reagents Co., Shanghai, China). Trizol, PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time), and SYBR® Premix Ex Taq™ (TliRNaSeH Plus) were purchased from Takara (Takara Bio, Kyoto, Japan). HPLC grade methanol and acetonitrile were obtained from Merck (Merck KGaA, Darmstadt, Germany). All other chemicals were of analytical grade.

**Plant Materials and Cell Culture Conditions**

*C. roseus* CMCs used in this research have been established and maintained in our research group as described previously.[19] CMC cultures were maintained at 25°C under continuous dark in MS solid media supplemented with 2% sucrose, 2.0 mg/L α-naphthylacetic acid (NAA) and 4g/L gelrite. Eight weeks prior to the experiments, 12-day-old CMC cultures were transferred to 250-ml Erlenmeyer flasks containing 100 mL MS solid media. The resulting cultures were added 2.0 mg/L NAA and cultivated at 25 °C with a 12/12-h light/dark photo period. Suspension cultures of CMCs were established by inoculating 12-day-old CMCs (5.0 g fresh weight) into 100 mL of fresh MS liquid media supplemented with 2% sucrose and 2.0 mg/L NAA, and were sub-cultured at 12-day intervals. Also, the suspension cultures were carried out on a HZT-2 gyrotory shaker (Donglian Electronic & Technol. Dev. Co., Beijing, China) with an agitation speed of 120 rpm at 25°C under continuous light. CMC growth was determined by grams of dry weight (DW) per liter.

Growth rate = (dry cell weight/initial dry cell weight) × 100%

**Inhibitor Treatment**

Lovastatin (200 mg) was dissolved in 7.5 mL of ethanol. After adding 11.25 mL of 0.1 M NaOH and incubating at 50 °C for 2h, the pH was adjusted to pH 7.2 with HCl, and distilled water was added to 50 mL to obtain a 10 mM stock solution of active lovastatin.[20] In the same as lovastatin solution was prepared, control solution was prepared just without adding lovastatin. Clomazone solution was prepared by dissolving 120 mg of it in 50 mL of 50% (v/v) ethanol to give a 20 mM stock solution, while control solution was 50% (v/v) ethanol.

Twelve-day-old suspensions of *C. roseus* CMCs were centrifuged at 300 × g for 10 min, and the media was discarded. CMCs (5.0 g fresh weight) were inoculated into 100 mL of fresh MS liquid media in 250-ml Erlenmeyer flasks at 25°C and 120 rpm under continuous light. After being filter-sterilized, lovastatin and clomazone solutions were added individually to 3-day-old suspension CMC cultures to give final concentrations of 10, 50, 100 and 150 μM, respectively. Control experiments were treated with corresponding blank solutions. Cells were harvested for 4, 6 and 8 days after treatment. The harvested cells were separated from liquid media by vacuum filtration, washed with distilled water, and freeze-dried. Experiments were performed in triplicate.

**Alkaloid Extraction and Determination**

The extraction of alkaloids from cells and liquid media was conducted according to a reported method.[21] The extracts were dissolved in 1.0 mL of methanol, filtered through 0.22-μm nylon membrane, and analyzed by HPLC. HPLC analysis was performed using an Agilent 1260 series system (Agilent Technologies, Santa Clara, CA, USA) equipped with a UV detector, an infinity quaternary pump and an autosampler. Chromatographic separations were performed by a Phenomenex Gemini C18 column (250 mm × 4.6 mm, 5 μm) (Phenomenex, Inc., Torrance, CA, USA) at 25°C. The mobile phase consisted of methanol/acetonitrile/10 mM ammonium acetate (15:40:45, v/v/v). The flow rate was set to 1.0 mL/min and the injection volume was 10 μL. The detection wavelength was 280 nm. MIAs were identified and quantified by comparing retention time and UV absorbance spectra with the commercial standards. Each sample solution was analyzed in triplicate.

**Monitoring Gene Expression by qPCR**

CMC cultures were frozen in liquid nitrogen and ground into the powder using a mortar and a pestle. Total RNA was extracted from CMC cultures according to the reported method.[19] RNA was quantified using a Nano Drop ND-2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Total RNA (1 μg) was treated with DNase to remove genomic DNA using a PrimeScript™ TM reagent kit with gDNA Eraser (Takara Bio, Kyoto, Japan), and then cDNA was synthesized according to manufacturer’s instructions (Takara Bio).

**Table 1:** The primer sequences for qRT-PCR

| C. roseus gene | Primer Sequence |
|---------------|-----------------|
| RPS9(AI749993) | TGAAGGCTTTTTGAGGAGGTG |
| Reverse primer | TGGCATCCCCAGACTTGAACA |
| Product size (bp) | 122 |
| DXX1(UC625536) | CGTGGGATGTTTGGCTCCTC |
| Forward primer | ATACCTGTGCGTCGCTCCTC |
| Reverse primer | 207 |
| Product size (bp) | DXX2(AJ011840) |
| Forward primer | AGGTGAGATCCCTTTTTTCTCC |
| Reverse primer | TTCTTGTGGCTTGCAATATTAG |
| Product size (bp) | 292 |
| DXX8(DQ8486762) | Forward primer |
| Reverse primer | GGCTGCGCTCTAATCCAAAG |
| Product size (bp) | TGTATTTTTTTTCAATTCCACA |
| DXXR(AF250233) | 230 |
| Forward primer | TCAAGCAGAAGCTGCTAATTC |
| Reverse primer | ACCAAATACAGAAGAAACCACACT |
| Product size (bp) | 159 |
| LAMT(EU0357974) | Forward primer |
| Reverse primer | GAGTAAATTTGATGCAGCCAAG |
| Product size (bp) | TTGATTGGATCAAGAATTGG |
| DTC(M25151) | 88 |
| Forward primer | TCCGAAAAACAGCCCATGCTG |
| Reverse primer | AAGGAGCGGTGGTGG |
| Product size (bp) | 126 |
| STR(X61932) | Forward primer |
| Reverse primer | TGACATTCGGCAGGCTTGG |
| Product size (bp) | CGCCGAGGAACTGATAGCTCT |
| D4H(U71605) | 122 |
| Forward primer | TACCCCTGATGCCCCTCAACC |
| Reverse primer | TTGAAGGCGCCCAATTGTG |
| Product size (bp) | 121 |
| ORCA3(AJ251250) | Forward primer |
| Reverse primer | CGAATTCATGGCCGAAAAAGC |
| Product size (bp) | CCTTATTCGGCCCAGGACT |
| 146 |
The transcript levels of 40S Ribosomal Protein S9 (RPS9, the housekeeping gene) and the concerned genes (DXS, DXS2A, DXS2B, DXR, TDC, LAMT, STR, D4H and ORCA3) were monitored. The primer sequences for RPS9, LAMT, TDC, STR, D4H, GES, ORCA3, SGD, DXS1, DXS2A, DXS2B and DXR[9,11,12] were shown in Table 1.

The qRT-PCR experiments were performed according to the SYBR® Premix Ex Taq™ (TliRNaseH Plus) kit protocol (Takara Bio.). Using the 96-wells thermal cycler (Bio-Rad, Hercules, CA, USA), all the qRT-PCR reactions were performed under the following conditions: 30 s at 95°C, and 40 cycles of 5 s at 95°C and 20 s at 60°C. Melt curve stage analysis (60°C–95°C) was used to verify the specificity of amplicons. The results of qRT-PCR analyses were subject to expression stability assay using Bio-Rad CFX Manager Software (Bio-Rad). All samples were measured in triplicate.

**Statistical Analysis**

All the values were reported as mean ± SD. Statistical analyses were performed using independent two-tailed Student’s t-test. All comparisons were made relative to untreated controls. Differences were considered significant at *p* < 0.05 (indicated by *; *p* < 0.01 indicated by **).

**RESULTS AND DISCUSSION**

**Effects of Lovastatin and Clomazone on the Growth of C. roseus Cambial Meristematic Cells**

Firstly, the growth curves of the untreated, lovastatin-treated and clomazone-treated C. roseus CMCs were made. Lovastatin and clomazone were added to 3-day-old suspension CMCs of C. roseus. After 4, 6 and 8 days, the CMCs were harvested and the dry cell weight of each group was recorded[Figure 1]. (Note: cell growth rate and the concerned MIA contents dramatically declined after 8-day incubation with enzyme inhibitors, so the longest incubation time was set to 8 days.) For all the groups, the cell weight reached to the maximum on the 8th day. In the presence of low-concentrated lovastatin (10 and 50 μM), the cells grew as well as the control groups did, but high-concentrated lovastatin (100 and 150 μM) dramatically inhibited cell growth as compared to the control groups, especially after day 6. Clomazone did not influence cell growth as much asLovastatin did. Forty six per cent and 67% reductions of cell growth were observed only in the presence of 150 μM clomazone on day 6 and 8, respectively. In order to exclude the possibility of MIA-production decrease caused by cell-growth inhibition, we focused our efforts on the effects of low-concentrated (10 and 50 μM) lovastatin and clomazone on accumulation of MIAs and transcription of their biosynthetic genes, whereas the effects of high-concentrated (100 and 150 μM) lovastatin and clomazone just served as the references.

**Effects of Lovastatin and Clomazone on MIA Gene Transcription in C. roseus Cambial Meristematic Cell Cultures**

Besides detection of MIA contents in C. roseus CMC cultures as mentioned above, the transcript levels of the MIA-biosynthetic genes encoding DXS, tryptonphan decarboxylase (TDC), loganic acid methyltransferase (LAMT), strictosidine synthase (STR), desacetoxyvindoline-4-hydroxylase (D4H) and ORCA3 (a jasmonate-
responsive transcriptional regulator) in the untreated and inhibitor-treated C. roseus CMCs were monitored in parallel by quantitative reverse transcription (RT)-PCR [Figures 3 and 4]. Among these enzymes, DXS may be derived from three genes, i.e. DXS1, DXS2A, and DXS2B. Low-concentrated lovastatin (10 and 50 μM) slightly increased the transcript amounts of DXS1 and DXR [Figure 3A and 3B] but didn’t show effect on DXS2A & 2B transcription (data not shown). However, lovastatin caused dramatic enhancement of the transcript levels of LAMT, TDC, STR, D4H, and ORCA3 compared with those of the control. Especially, in the presence of 50 μM lovastatin, the maximal relative transcript levels of TDC, LAMT, STR, D4H and ORCA3 were 3.1, 2.3, 2.8, 3.4 and 4.0 times higher than those of the control, respectively [Figure 3C–G]. Although it was unclear that how much the transcription of HMGR in the CMCs was reduced by lovastatin due to the lack of the knowledge of HMGR in C. roseus, it is apparent that the inhibition of HMGR doesn’t decrease accumulation of MIAs, confirming that the MEP pathway is the major source of IPP used for biosynthesis of MIAs. Inhibition of HMGR might cause a global deficiency of IPP and DMAPP in cells, which, together with the crosstalk between MVA and MEP pathways, could lead to the slight increase of the transcription of DXS1 and DXR to overcome the IPP deficiency when the CMCs were treated with 10 and 50 μM lovastatin [Figure 3A and 3B]. The enzyme DXS is mainly derived for DXS2A & 2B according to the previous report, the treatment of lovastatin however had no impact on the transcription of DXS2A & 2B (data not shown). Therefore, enhancement of MIA accumulation in lovastatin-treated groups is not due to the increase of DXS1 mRNA level. Even if the higher DXS1 mRNA level caused by lovastatin brought into a bit of excess accumulation of IPP, it is unreasonable that the transcription of TDC, LAMT, STR and D4H was simultaneously up-regulated because these genes located at the downstream steps of IPP which could inhibit their transcription. The transcription of ORCA3 and the concerned MIA-

Figure 2: Effects of lovastatin and clomazone on production vindoline (A1 and A2), catharanthine (B1 and B2) and ajmalicine (C1 and C2) in C. roseus CMCs. Values are means ± SD of triplicate experiments. Data were analyzed by ANOVA followed by Student’s t test. Significant differences between treatments and the control are shown as $p < 0.05$ (*) and $p < 0.01$ (**).
biosynthetic genes was almost synchronously induced by lovastatin except that only the maximal induction to $TDC$ shifted slightly in time and occurred on day 8 in the presence of 50 M lovastatin, but the magnitude on day 6 was very close to that on day 8 [Figure 3C–G]. Therefore, we reasoned that the transcription of $TDC$, $LAMT$, $STR$ and $D4H$ was activated by the increase of ORCA3 transcript level which was induced by lovastatin via an unknown mechanism. This hypothesis is also consistent with the fact that ORCA3 manipulates the transcription of $TDC$, $STR$, $SGD$ and $D4H$.\[23-26\]

Four days after the treatment of clomazone, the transcript level of $DXS1$ declined and $DXS 2A$ & $2B$ mRNA levels dramatically increased [Figure 4A–C], which was consistent with the reported results.\[15\] And the transcript levels of $TDC$, $LAMT$, $STR$, $D4H$ and ORCA3 decreased [Figure 4E–I], which could be the reason that led to decline of MIA accumulation. [Figure 2A2, B2 and C2]

Figure 3: Effects of lovastatin on expression of MIA genes in C. roseus CMCs. Values are mean ± SD of triplicate experiments.

Figure 4: Effects of clomazone on expression of MIA genes in C. roseus CMCs. Values are mean ± SD of triplicate experiments.

CONCLUSIONS

In summary, the present study confirmed that $DXS 2A$ & $2B$ mainly contributed to the production of isoprenoid IPP which were used for biosynthesis of MIA. HMGR inhibitor lovastatin and $DXS1$ inhibitor clomazone not only influence the production of IPP and DMAPP, but also cause evident effects on transcription of downstream genes. This indicates that biosynthesis of MIA is manipulated
via a complex mechanism, thus MIA accumulation depends on the comprehensive effects caused by the alteration of the transcription of their biosynthetic genes. These findings pave the way for rationally tuning metabolic flux to improve MIA production in C. roseus CMCs.

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