Sun Pharma Science Scholar Awards

Title: Metabolic engineering strategies for enhanced production of anti-cancer drug – camptothecin, using plant cells of *Nothapodytes nimmoniana*

Introduction

Camptothecin (CPT), a monoterpene indole alkaloid is the third most sought-after drug used in anti-cancer therapeutics. The primary natural source of CPT is from the plants, *Camptotheca acuminata* and *Nothapodytes nimmoniana* (native to South-east Asia). Utilization of natural plants as a source for pharmaceutical products has been an arduous task due to widely varying seasonal and soil conditions, extensive deforestation and habitat loss rendering these plants in the endangered species category. To meet the increasing market demand, plant cell/tissue culture can be used for germplasm conservation, micro-propagation of high-yielding plants and also as sustainable source for *in vitro* production of CPT at large scale. Understanding the complex metabolic networks of plants to produce secondary metabolites like CPT can be achieved by the construction of comprehensive metabolic models to determine plant phenotypes and predict plant metabolic behaviour, facilitating the selection of suitable strategies, saving time and cost over a traditional hit-trial approach in experimentation. Hence, we propose to reconstruct a genome-scale metabolic model using constraint-based modelling methods, identify targets for gene-overexpression and experimentally validate them for enhanced CPT production in cell lines of *N. nimmoniana.*

Objectives

1. Reconstruction of a Genome scale metabolic model (GEM) for camptothecin production
2. Identifying targets for over-expression/knock-outs to improve camptothecin productivity
3. Development of a high yielding genetically transformed cell lines of *N. nimmoniana* via *Agrobacterium* mediated transformation
4. Overexpression of strictosidine synthase in *N. nimmoniana* plant cells to enhance camptothecin production

Material and Methods

The objective of this study was to develop high-CPT yielding cell lines of *N. nimmoniana* using rational metabolic modelling. The first step involved the reconstruction of a genome-scale metabolic model for CPT production. Owing to the unavailability of *N. nimmoniana* whole-genome sequence, the whole genome sequence of *C. acuminata*, another CPT-producing plant native to China and Eastern Asia which was available was chosen for the study. A draft metabolic model was generated using the *C. acuminata* whole genome sequence (Zhao et al. 2017) in ModelSEED, a resource for reconstruction, comparison, and analysis of metabolic models (Henry et al. 2010). From the whole genome sequence, the *C. acuminata* protein FASTA sequence was procured and uploaded to https://modelseed.org/plant using the "Upload Plants FASTA" option (Seaver et al. 2018). The COBRA toolbox 3.0 (Schellenberger et al. 2011) for MATLAB (version R2019b, MathWorks Inc.) was used for constraint-based modelling and analysis of the metabolic model.

To reconstruct a metabolic network for CPT biosynthesis **(Fig. 1)**, organism-specific biochemical data from literature and different databases such as Plant Metabolic Networks (PMN) (Schläpfer et al. 2017) and Kyoto Encyclopedia of Genes and Genomes (KEGG) were collected. In addition, recently a transcriptome analysis in *N. nimmoniana* had also been reported, unravelling 13 genes associated with CPT biosynthesis (Manjunatha et al. 2016). A Basic local alignment search tool (BLASTP) analysis performed between the two plants viz., *N. nimmoniana* and *C. acuminata* demonstrated similarity between them due to homology in the camptothecin-producing genes. Extensive manual curation was performed following standard procedures (Thiele and Palsson 2010), including - gap filling, maintenance of reaction directionality, uniformity in the nomenclature of identifiers, updating metabolite and reaction details, including some biosynthesis reactions of CPT in accordance with the PMN database, adding missing formulae and charges to the metabolites, fixing reaction balances, reaction duplicates and stoichiometries. We also identified metabolic dead-ends and blocked reactions in the model.

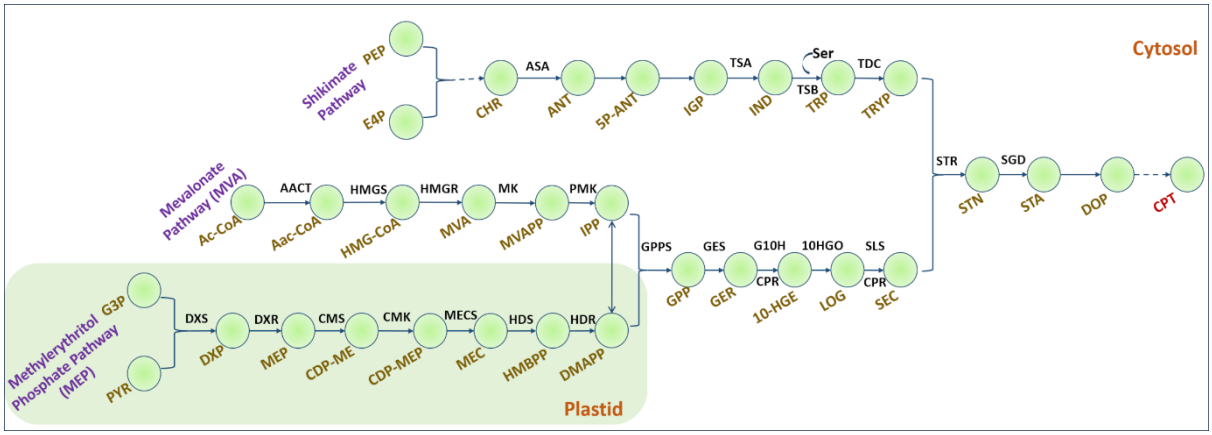


Fig. 1 Camptothecin biosynthesis in plants
Dashed lines indicate multiple steps. Shikimate pathway: PEP – Phosphoenol pyruvate; E4P – Erythrose-4-phosphate; CHR – Chorismate; ASA – Anthranilate synthase; ANT – Anthranilate; 5P-ANT – 5-Phosphoribosyl anthranilate; IGP – Indole glycerol phosphate; TSA – α-subunit of tryptophan synthase; IND – Indole; Ser – Serine; TSB – β-subunit of tryptophan synthase; TDC – Tryptophan decarboxylase; TRYP – Tryptamine. Mevalonate pathway: Ac-CoA – Acetyl Coenzyme A; AACT – acetyl CoA: acetyl CoA C-acetyltransferase; AaC-CoA – Acetoacetyl CoA; HMGS – 3-hydroxy-3-methylglutaryl-CoA synthase; HMGR – 3-hydroxy-3-methylglutaryl-CoA reductase; MK – mevalonate kinase; PMK – phosphomevalonate kinase. Methylerythritol phosphate pathway: G3P – glyceraldehyde-3-phosphate; DXS – 1-deoxy-D-xylulose-5-phosphate synthase; DXP – 1-deoxy-D-xylulose- 5- phosphate; DXR – DXP reductoisomerase; MEP – 2-C-methyl erythritol-4-phosphate; CMS – 4-(cytidine 5-diphospho)-2-C-methylerythritol synthase; CMK – 4-(cytidine 5-diphospho)-2-C-methylerythritolkinase; MECS – 2-C-methylerythritol-2,4-cyclodiphosphate synthase; HDS – hydroxymethylbutenyl 4-diphosphate synthase; HDR – hydroxymethylbutenyl 4-diphosphate reductase; HMBPP – hydroxy methyl butenyl diphosphate; IPP – Isopentenyl diphosphate; DMAPP – dimethylallyl diphosphate; GPP – geranyl diphosphate; GPPS – GPP synthase; GES – geraniol synthase; GER – Geraniol; G10H – geraniol 10-hydroxylase; CPR – NADPH – cytochrome P450 reductase; 10-HGE – 10-hydroxy geraniol; 10-HGO – 10-hydroxy-geraniol oxidoreductase; LOG – Loganin; SLS – Secologanin synthase; SEC – Secologanin; STR – Strictosidine synthase; STN – Strictosidine; SGD – Strictosidine beta-glucosidase; STA – Strictosamide; DOP – Deoxypumiloside; CPT – Camptothecin

During model reconstruction, a biomass formation reaction was generated, which was validated experimentally. To formulate the *N. nimmoniana* specific biomass objective function (BOF) and determine the biomass precursors, the cell suspension cultures of *N. nimmoniana* were lyophilized and analyzed. Cell biomass samples (in replicates, n=4) were

used for the metabolite analysis using Gas-chromatography mass spectrometry (GC-MS) facility (Agilent Technologies, USA). The biomass precursors were divided into protein, DNA, RNA, carbohydrates, lipids, cell wall components, inorganic ions, and metabolites.

Flux Balance Analysis (FBA) (Varma and Palsson 1994; Raman and Chandra 2009; Orth, Thiele, and Palsson 2010) is used to calculate the flow of metabolites through a metabolic network and enables us to predict the organism's growth rate and the rate of production of biotechnologically important metabolites. In our model, FBA was used to analyze NothaGEM by constraining exchange reactions in the model with experimental values of the substrate and metabolite uptake rates. FBA was performed with the objective function of maximizing cell biomass, and the model-predicted specific growth rate was estimated.

Flux scanning based enforced objective flux (FSEOF) was then used to identify targets for over-expression/knock-outs and rank them for higher camptothecin productivity (Choi et al. 2010). FSEOF scans for those reaction targets with monotonically increasing fluxes when camptothecin formation is enforced in steps while maximizing growth as the objective function.

Results

A genome-scale metabolic model for camptothecin production was reconstructed by incorporating available genome data into ModelSEED. The draft model comprised 1162 metabolites and 1104 reactions, including central carbon metabolism and tryptophan biosynthesis. The reaction directionality was modified based on information available in the Plant Metabolic Network database (PMN) (https://plantcyc.org/organism-summary?object=CACUMINATA). The nomenclature of the metabolites/identifiers were maintained uniformly from KEGG (Kyoto Encyclopaedia of Genes & Genomes).

Twenty camptothecin biosynthesis reactions were added to the model, out of which 18 reactions were directly involved in the CPT pathway, one transport reaction of tryptamine from the stroma to the cytosol and one exchange reaction to account for the secretion of camptothecin. Dead-end metabolites and blocked reactions were removed, specifically from the stroma and the cytosolic compartments, since the biomass production and camptothecin biosynthesis were focused in these two compartments. Some important metabolites like beta-carotene, ascorbate, and vitamin E were detected as dead-ends. As a gap-filling step, their corresponding biosynthesis and metabolic reactions were added to the model. Overall, the total blocked reactions were reduced to 299 from 385, and the dead-end metabolites were reduced to 413 from 507. GC-MS analysis of *N. nimmoniana* cells and suspension culture filtrate revealed the presence of mannitol, malate, and oxo-proline. Hence, reactions associated with them were incorporated into the model to improve the predictive capability of the model. Stoichiometrically inconsistent reactions were identified and replaced with balanced, consistent reactions to make the model entirely stoichiometrically consistent with mass and charge balance and no duplicate reactions. The final model, NothaGEM *i*SM1809, comprised 1228 metabolites, 1233 reactions, and 1809 genes.

The model is compartmentalized into 11 compartments, namely –cytosol, stroma, golgi apparatus, vacuole, cell wall, peroxisome, mitochondria, etc. There are 23 exchange reactions and 296 transport reactions, indicating high metabolite interconnectivity among the intracellular compartments and 914 metabolic reactions.

We used FSEOF to screen and identify over-expression and knock-out targets for camptothecin production. Fifty-five reactions were identified and ranked among the over-expression targets. STR was one of the top-ranked enzymes. STR catalyzes the conversion of tryptamine and secologanin to strictosidine, an important mono-terpene indole alkaloid intermediate in camptothecin biosynthesis. The main criteria for selection of STR for over-expression was its strategic location and thereby, it would have a more direct effect on camptothecin biosynthesis. This model predicted STR synthase over-expression was experimentally implemented using *Agrobacterium*-mediated transformation. Among the other top-ranked targets predicted, CPT biosynthesis pathway reactions, including mevalonic acid pathway and shikimate pathway, amino acid metabolism – methionine, and nucleotide biosynthesis, were also predicted but not experimentally validated. The model predicted some new leads for over-expression targets like succinate and citrulline. This is concurrent with literature where succinic acid has been used to enhance terpene alkaloid production three-fold in *Catharanthus roseus* (Changxing et al. 2020).

Among the top-ranked targets for knock-outs, reactions involving the formation of folates, serine, and conversion of acetyl CoA and oxaloacetate to malate and citrate have been predicted. Isomerization reactions involving the conversion of 3-phosphorglycerate to phosphoenolpyruvate and glyceraldehyde-3-phosphate to glycerone phosphate have also been predicted.

The model predicted top-ranked target STR was used to generate engineered cell lines of *N. nimmoniana. In vitro* grown shoots, leaf, and petiole regions of *N. nimmoniana* were transformed using *A. tumefaciens* LBA4404 with pCAMBIA 1301 containing the At-STR cDNA. Fifteen transformed cell lines were induced after four months of transformation, out of which four cell lines were sustainably growing after ten subcultures. The transgenic expression was confirmed using GUS analysis, PCR, and qPCR **(Fig. 2).**

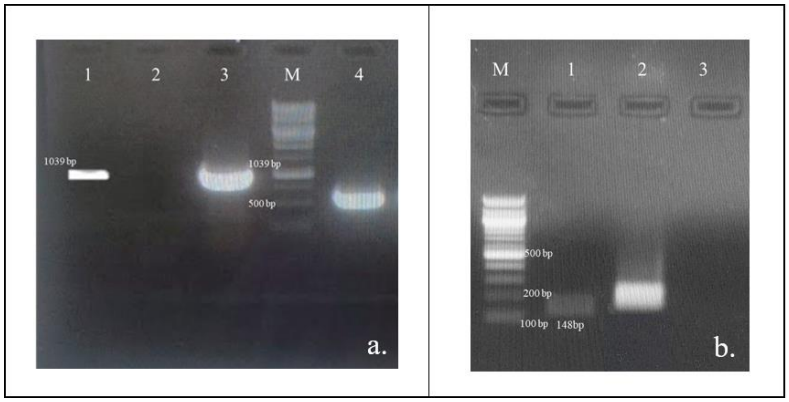


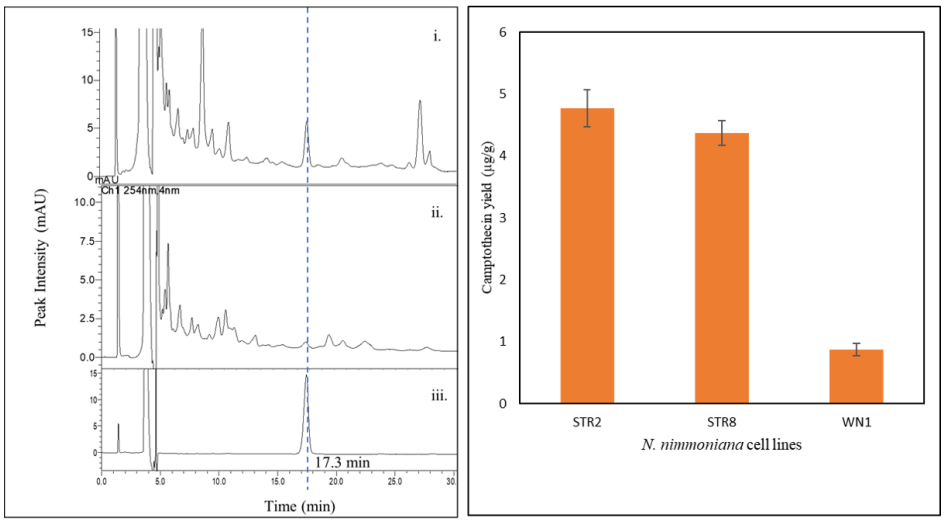
Fig. 2 – Verification of the At-STR gene integration and expression;

a - PCR amplification of At-STR gene (1039 bp) from the transformed cell lines of *N. nimmoniana* visualized on 0.1% agarose gel. Lane 1: plasmid DNA of *A. tumefaciens* LBA4404 harbouring At-STR construct of 1039 bp (positive control), Lane 2: Untransformed cell line, WN1 (negative control), Lane 3: PCR product of transformed cell line(STR2) showing amplification corresponding to At-STR (1039 bp), Lane M: 1 Kb ladder, Lane 4: PCR product (STR2) showing GUS expression (500bp);

b - Verification of At-STR transgenic expression in untransformed and transformed cell line STR2 using semi-quantitative RT-PCR followed by visualization of the amplified products on a 1% agarose gel Lane M: 100

bp ladder, Lane 1: At-STR expression in *N. nimmoniana* (148 bp), Lane 2: *Nn*-Actin (Positive internal control), Lane 3: Water (negative control)

CPT was extracted from the transformed and untransformed cell biomass and estimated using a RP-HPLC. **Fig. 3** shows the CPT chromatogram and yield obtained from the cell lines of *N. nimmoniana* from the HPLC analysis. CPT yield of 4.77 µg g^{-1}, 4.37 µg g^{-1} and 0.87 µg g^{-1} were obtained from *N. nimmoniana* over-expressed cell lines STR2, STR8, and the wild-type cell line (WN1) respectively.



a. b.

Fig. 3 a– HPLC chromatogram of CPT detected in various samples - i. Metabolically engineered *N. nimmoniana* callus harbouring At-STR; ii. Wild-type *N. nimmoniana* callus; iii. Standard Camptothecin; b. Camptothecin yield from callus cultures of *N. nimmoniana.* STR – strictosidine synthase callus, WN – Wildtype *N. nimmoniana* callus

Discussion

Increasing market demand for camptothecin production has led to excessive deforestation, endangering the *N. nimmoniana* plant species in India. Additionally, being a tree species, it has a slow growth rate and typically requires 7-8 years before it can be harvested for the first time. Alternative methods for *in vitro* production of camptothecin in bioreactors using plant cell suspensions and the development of high-yielding plant varieties are the need of the hour to prevent the depletion of natural sources that will be efficient, eco-friendly, and cost-effective to sustainably produce CPT on a large scale. In this regard, rationally designed metabolic engineering strategies can be used to generate such high-yielding plant cell lines.

Hence, to this effect, we reconstructed a genome-scale metabolic model for CPT production. The draft model generated from ModelSEED represents different metabolic pathways in plants, including the central carbon metabolism. Since sucrose was the primary carbon source used to grow *N. nimmoniana*, our initial steps involved analyzing the model for the production of biomass, step-wise from sucrose, and other media components. While we could account for biomass production, the production of CPT was also an essential objective of our work. To achieve this, we added reactions corresponding to CPT biosynthesis involving the shikimic acid pathway, mevalonic acid pathway, and the methylerythritol

pyrophosphate pathway systematically in their respective compartments as reported by (Rather et al. 2018; Murali, Rajendran, and Srivastava 2021). While performing FBA, we observed an increase in biomass flux led to a decrease in the camptothecin flux, and vice-versa. This demonstrated the known fact of camptothecin being a secondary metabolite, increasing confidence in the predictive power of the drafted metabolic model.

Among the top-ranked predicted over-expression targets, the CPT biosynthesis pathway genes included STR, tryptophan decarboxylase (TDC), geraniol 10 hydroxylase (G10H), tryptophan synthase, and mevalonate oxidoreductase. Over-expression of TDC and STR individually and synergistically has been reported in *C. roseus* by Canel et al. 1998, where STR over-expression showed 10-fold higher STR activity than in the wildtype cultures (Canel et al. 1998). Similarly, over-expression of G10H in *C. roseus* hairy roots has been reported to enhance catharanthine production (Wang et al. 2010). The co-overexpression of G10H and STR has reportedly improved CPT content in the hairy roots of *Ophiorrhiza pumila* (Cui et al. 2015). STR and G10H from *C. roseus* were individually and simultaneously introduced into *O. pumila.* Overexpression of only G10H significantly improved CPT production compared to the non-transgenic hairy root culture, implying that G10H could play a vital role in CPT accumulation in *O. pumila.* These studies have also shown a significant increase in CPT production where co-overexpression of G10H and STR has demonstrated an increase in the yield of CPT by 56 % as compared to single overexpression lines and non-transgenic lines (Cui et al. 2015).

Some novel reactions, uninvolved in the upstream CPT biosynthesis have also been predicted by our approach as targets for over-expression. These targets include reactions involving the production of homocysteine, succinate, citrulline, and 5-oxoproline. Homocysteine is known to produce methionine, a vital amino acid that initiates protein synthesis. Homocysteine metabolism predicted as one of the over-expression targets, has also been reported to be influenced by environmental stress, which can presumably enhance secondary metabolism. Succinic acid, another target predicted, has been used to enhance terpene alkaloid production in *C. roseus*. In a study by Changxing et al., addition of 10 mM succinic acid as a precursor to *C. roseus* callus cultures enhanced serpentine and ajmalicine production by four and five fold, respectively (Changxing et al. 2020). Citrulline, a critical intermediate in the arginine pathway, and 5-Oxoproline producing glutamate were also predicted as CPT over-expression targets. Under stress conditions, the synthesis of abundant amino acids such as proline, glutamate, arginine, and asparagine are upregulated. These amino acids act as compatible osmolytes and precursors for secondary metabolite production and hence, can be used for increasing CPT production.

Among the knock-out targets predicted, 24 reactions were identified, as mentioned in Table 1b. Two reactions involving the formation of folates, viz., methyl tetrahydrofolate hydrolase and methylenetetrahydrofolate oxidoreductase, were predicted as knock-out targets. While it is well known that folates play a vital role as cofactors, it is intriguing to note that they are produced from the shikimate pathway, where chorismate is converted to p-aminobenzoate, which goes on to synthesize folates. When this branched pathway is blocked, it can lead to increased amounts of chorismate, leading to an increase in CPT production. Similarly, the conversion of serine to phosphoserine has been identified as a knock-out target. In CPT biosynthesis, serine plays an important role in the conversion of

indole to tryptophan (**Fig. 1**). Downregulating the conversion of serine to phosphoserine can lead to enhanced production of tryptophan and, thereby, CPT.

To our knowledge, this is the first report on enhanced camptothecin production from a metabolic engineering strategy in *N. nimmoniana* callus cultures, where a five-fold increase in CPT production has been obtained. The production of CPT was confirmed by HPLC, and the overexpression was confirmed using PCR and qRT-PCR.

Impact of the research in the advancement of knowledge or benefit to mankind

Development of sustainable plant cell bio-factories for mass production of value-added molecules holds the possibility to expand access to healthcare and meet UN's Sustainable Development Goals (SDGs) and the World Health Organisation's (WHO) directive on promotion of herbal medicines among the low-income population worldwide. The pharmaceutical potential of phytochemicals render them the potent drug candidates for the alleviation of several health disorders and the large-scale production of these metabolites economically is a pressing priority. Numerous phytochemicals have industrial significance in addition to their uses in medicine since they are also used in supplements, cosmetics, and other products. Most importantly, sustainable biomanufacturing based on plant cell bio-factories can promote bioeconomy, which has become crucial for phasing out fossil resources and to meet the climate change mitigation goals via biodiversity conservation.

In this study, we successfully enhanced camptothecin yield in *N. nimmoniana* plant cells through a model-driven metabolic engineering strategy. A genome-scale metabolic network – NothaGEM *i*SM1809, was reconstructed for camptothecin production in *N. nimmoniana* cells and curated by integrating experimental data. *In silico* constraint-based modelling approaches were then used to predict strategies for further enhancement of CPT. Similar to the STR-mediated over-expression strategy, other predicted enzyme targets can also be experimentally implemented to enhance CPT production. Prediction of non-intuitive targets like citrulline and succinic acid depict the predictive power of the model. These targets can be also be experimentally implemented in combination or alone for further enhancement in CPT yield. They can be applied to choose the most stable callus lines for process optimization and large-scale production of industrially important metabolites.

To our knowledge, this is the first report on a metabolic model that has been developed for the production of camptothecin. CPT is produced from strictosidine, a crucial intermediate in plant indole alkaloid biosynthesis. The model can be extended to produce other commercially important and pharmaceutically valuable phytochemicals that can be synthesized from strictosidine. Some well-known secondary metabolites produced from strictosidine include quinine which is commonly used to treat malaria and babesiosis, serpentine and ajmalicine used to treat hypertension, vinca alkaloids - vincristine, and vinblastine used in anti-cancer therapeutics. Using model-predicted strategies, high-yielding plants and cell-culture systems can be generated for *in vitro* production of metabolites at a large scale in bioreactors.

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