Celastrol Biotransformation via Recombinant Glycosyltransferases for the Potential Use of Cancer Treatment

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ABSTRACT

Many of the herbal secondary metabolites are broadly used in the field of medicine, most of which are triterpenoids. Celastrol is one of the compounds that belong to the triterpenoid family and its utilisation in the treatment of various diseases is highly investigated. However, its high toxicity restricts its use in the pharmaceutical field. Glycosyltransferase enzymes catalyse the glycosylation reactions, which leads to a decrease in the toxicity of celastrol. For this purpose, it was aimed to decrease the toxicity of celastrol through the biotransformation via glycosyltransferase enzymes using synthetic biology approaches. As an output, it is expected to obtain glycosylated celastrol which has lower toxicity than native celastrol. The importance of this study is the impact of celastrol on many vital diseases such as cancer, obesity, Alzheimer's disease, and Parkinson's disease.

Key Words: Celastrol, glycosyltransferase, biotransformation, glycosylation, toxicity, cancer treatment, synthetic biology, triterpenoid

INTRODUCTION

The celastrol molecule is a molecule in the triterpenoid class with 29 carbon atoms. Its formula is C29H38O4 and its molecular weight is 450.6 Da (Amirruddin et. Al., 2018). It is obtained from a plant called *Tripterygium wilfordii*, which grows only in China today. Although the plant itself is poisonous, the root of the plant is a source of various biologically active compounds (Salminen et. Al., 2010). Although it has been used in traditional medicine in China, it is not used today due to its toxic effect for very low doses.

Furthermore, celastrol is one of the most potential herbal molecules isolated from plant extracts to be used as a medicinal product (Lu et. al., 2021). Celastrol has great potential for use in the pharmaceutical industry due to its bioactive nature, anti-cancer, anti-inflammatory activity and anti-obesity effects (Lu et. al., 2021). Due to its potential therapeutic effects and unique chemical structure, it has attracted great attention not only in terms of biosynthesis but also in medicinal chemistry and organic fields (Lu et. al., 2021).

According to recent studies, celastrol's potent anti-inflammatory (Kim et. al., 2009; Nakamichi et. al., 2010) and neuroprotective effects (Cleren et. al., 2005) have been investigated for the treatment of neurodegenerative diseases such as Parkinson's and Alzheimer's (Allison et. al., 2000), where an elevated inflammatory environment is observed around neuron cells with over-activa-

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ted microglia. Moreover, in laboratory experiments, celastrol has been proven to be a potent inhibitor of inflammatory responses and cancer formation, as well as alleviating proteostasis deficiency diseases (Salminen et. al., 2010). Studies have shown that celastrol can potentially be used for the prevention and treatment of cancer by inhibiting proteasomes (Yang et. al., 2008). However, due to celastrol's toxic effects and suboptimal pharmacological properties, its clinical applications remain limited. Likewise, it was seen that celastrol's toxic effects on embryonic development and cardiotoxicity have been observed in zebrafish, even in low concentrations (Bai, Fu, Zhang, Yue, Chen, Xu, & Tang, 2021). Therefore, further toxicity assessment of celastrol is required prior to clinical trial (Wang et. al., 2011; Liu et. al., 2019; Shan et. al., 2013).

Multiple studies using different model organisms, such as zebrafish embryos, mice, and spermatogenic cells, have suggested that celastrol may cause infertility, liver toxicity, and toxic effects on embryo development (Wang et.al., 2011). Even though celastrol has been found to prevent the formation of tumours in human glioblastoma cells by causing proteotoxic stress, which changes the signalling of cells, it may also disrupt the normal development of healthy cells and lead to unwanted cell death (Brancolini, & Iuliano, 2020). It is known that the relatively high concentrations of celastrol (0,5 mg/kg/day, i.p.) affects blood pressure and basal metabolism, additionally, 5 and 7,5 mg/kg/day concentration of that molecule was found enough to disrupt the gut structure in mice (Chang, Wang, Chiang, Li, & Chen, 2020).

Despite the fact that its toxicity was reduced with recent studies, it may still be used in the treatment of some vital diseases like cancer, obesity, and HIV due to that high toxicity. To achieve that, celastrol has to be structurally modified before use. Another reason to modify that molecule is its low bioavailability and stability (Shi, Li, Xu, Chen, Luo, Zhang, & Fu, 2020). It is possible to create more soluble and less toxic derivatives of celastrol by biotransformation. The pharmacologically stronger properties of the modifications made on different C atoms indicate that optimization is possible and that these celastrol derivatives have a positive effect in terms of possible clinical use.

Various modifications can be made to improve limitations such as low solubility using celastrol and make it more active. As recent studies have revealed, some glycosyltransferase enzymes are obtained from celastrol while reducing toxicity (Ng et al., 2019). Chang et al. in their research, also showed that the toxicity of celastrol is reduced when modified, both in the *Bacillus subtilis* species have the potential to increase the water solubility and activity of *in vivo* and *in vitro*. In this study, celastrol was modified by using the glycosyltransferase enzyme encoded as BsGT110 of *Bacillus subtilis* organism and celastrol-29-O-β-glucoside molecule was obtained. With the addition of this glucose to C-29 of celastrol, its water solubility has increased approximately 50 times, resulting in an increase in survival rate from 0% to approximately 80% (Chang et al., 2021).

In a study, it was found that celastrol is able to influence Activating Transcription Factor 2 (ATF2) peptide transcription, which can induce apoptosis in mouse and human melanoma cells. By *in vitro* analysis of the series of C-20 amid/ester derivatives such as pristimerin and dihydrocelastrol (DHCE), it was detected that pristimerin has similar activity to celastrol whereas DHCE which

does not have a quinone methide structure and shows no activity. For this reason, it was concluded that the celastrol molecule may play a role in apoptotic activity (Veerappan et al., 2017).

In another study, modifications in C-3 of celastrol were compared to analyse their neuroprotective activity. The carboxylic group modifications in C-3 developed high protection at concentrations of 0.6 – 1.8 μM as amide derivatives, while the ester derivatives were not so high in terms of protection against toxic and inflammatory effects in the nervous system. (Allison et al., 2000).

Apart from *in vivo* or *in vitro* experiments, to look at the communication between proteins and possible ligands that will interact with them, using *in silico* applications, that is, computer programs capable of protein-ligand interaction analysis, accelerates the studies and gives a great privilege in choosing the most likely situation to get results. Thanks to such programs, studying the interactions of possible ligands with different proteins provides great convenience in situations such as predicting experimental data in advance and verifying these data.

In conclusion, celastrol is a potential drug, but its high toxicity even at low concentrations prevents its applicability of celastrol as a drug for the treatment of diseases that have a high mortality rate like cancer, obesity, Alzheimer's disease, and cardiovascular diseases. Therefore, we aimed for a human-centered design to modify the celastrol molecule by glycosyltransferase enzyme to improve its solubility and reduce toxicity. Additionally, since the celastrol modification studies are very limited in the literature, modification studies will fill the gap for further research. In this study, we designed an experiment to examine the interaction between celastrol and the potential "glycosyl transferase" enzymes that have been chosen by "Molecular Docking" and "Molecular Dynamic Simulations" programs as the first sub-aim. After the selection of appropriate glycosyl transferase enzymes by these programs, the experiment will continue with the *in vitro* modification of the celastrol molecule, and the toxicity assays onto the defined cell cultures will be analysed, which involves the second and third sub-aims. In this way, the usage of celastrol will be increased considerably in the medical and pharmaceutical industries. Additionally, studies on the modification of celastrol have the potential to support further celastrol studies and possibly fill the research gaps in academics.

MATERIALS AND METHODS

Molecular Dynamic Simulations. Initially, optimization of Celastrol and Uridine-5'-Diphosphate-2-Deoxy-2-Fluoro-Alpha-D-Glucose will be performed via Gaussian software by using the B3LYP method and 6-31+G** basis set in the diethyl ether solvent. After geometry optimization, celastrol will be docked to the active site of the glycosyltransferase enzyme by using Autodock software. Then, Molecular Dynamic Simulations will be performed both for the monomer and dimer structure of the protein. After simulations will be performed, to calculate the binding free energies of the system MM/PBSA (The Molecular Mechanics/Poisson-Boltzmann Surface Area) method will be used. Finally, by using Thermodynamic Integration, further energy calculations will be performed and analyses will be done.

Recombinant Production of Glycosyltransferase. Plasmid design will be made by searching the relevant gene sequence encoding the glycosyltransferase protein from *Bacillus thuringiensis* in the NCBI gene database. Then, the designed insert by optimising according to the host organism and vector backbone (pDEST-periHisMBP (AddGene)) will be inserted. The reason for selecting this vector backbone is it has a signal sequence that allows the target protein to be localized to the periplasm, which may promote disulfide bond formation and reduce toxicity, and also has a 6xHis tag

for the purification of the protein of interest. BamHI and HindIII restriction enzymes were added to the 5' and 3' end of the insert, respectively, because of zero cutters. Then, the insert will be ligated to the expression vector backbone with T4 ligase. The chemical transformation process will be done by transferring the plasmid to the *Escherichia coli* DH5α competent cells via heat shock. An equal volume of plasmids and competent cells will be mixed in a microcentrifuge tube and the mixture will be incubated on ice for 20-30 minutes. After incubation, the transformation will be done by heat shock applied at 42°C for 45 seconds. As the cells will be transformed, 100 µL of transformants will be inoculated in an LB agar medium containing kanamycin antibiotic and will be incubated at 37°C in an LB agar medium with kanamycin antibiotic overnight. After incubation, single colonies will be taken and protein expression of the transformant will be induced in LB broth media containing kanamycin antibiotic, and the glycosyltransferase protein will be purified with the ammonium sulphate precipitation method and His-Tag method.

Ammonium Sulphate Precipitation and His Tag Purification. 65% saturated ammonium sulphate solution will be prepared by heating gently until all of the ammonium sulphates will be dissolved and letting it cool to working temperature on a magnetic stirrer. After crystals of ammonium sulphate will be formed, either the appropriate volume of saturated ammonium sulphate or solid ammonium sulphate will be added to the sample to get the desired concentration. The final solution will be stirred for 1 hour to fully equilibrate and will be centrifuged at 10,000 × g for 15 minutes to pellet out the protein. Finally, pellets will be dissolved in PBS to analyze proteins. To isolate the enzyme, the protein will be precipitated after standing at 4°C for 8 hours, and the supernatant will be discarded after centrifugation for 15 minutes at 17,700 × g. The precipitated protein will be redissolved with an appropriate volume of phosphate buffer (pH= 7.0), then will be placed in a dialysis membrane and will be dialysed against the buffer at 4°C for 24–36 hours. The dialysate will be centrifuged at 4°C with a rotation speed of 17,700 × g for 5 min, and then the supernatant (enzyme solution) will be collected. The sample will be filtered (0.22 µm) and will be loaded onto a DEAE-Sepharose FF column will be pre-equilibrated with the phosphate buffer. The column will be eluted using a linear gradient of phosphate buffer used at the beginning of the process and to another phosphate buffer with a 1 mL/min flow rate. Histidine-linked glycosyl transferases will be isolated using HisTrap™ High Performance according to the manufacturer's protocol.

SDS-Page Gel Electrophoresis. The SDS-PAGE will be performed by using 5% stacking gel and 12% separating gel to analyse whether the glycosyltransferase is correctly produced or not. BenchMark™ protein ladder will be used as a molecular weight standard.

Activity Assay for Isolated Glycosyltransferase. The enzymatic experiments will be used a total reaction volume of 50 µL, 50 mM Tris HCl (pH 7.5), 100 M UDP-glucose, various substrate concentrations (dissolved in DMSO), and 5 g of pure UGT72B27. All enzymatic reactions will be carried out in Eppendorf tubes using incubators at 30 °C for 10 minutes with 400 rpm shaking. Overall the activity test for the glycosyl transferase will be performed using the Glycosyltransferase Activity Kit, reference EA001 - R&D, Wiesbaden, Germany.

Biotransformation of Celastrol. To transform the celastrol with recombinant glycosyltransferase with Uridine-5'-Diphosphate-2-Deoxy-2-Fluoro-Alpha-D-Glucose (U2F-Glucose) *in vitro*, recombinant glycosyltransferase will be purified and a 100 µL reaction will be set up. With 20 mg/mL of glycosyltransferase, 1 mM of U2F-Glucose and 50 mg/L of celastrol reaction will be conducted in 10 mM of MgCl2, 5% DMSO and 50 mM of Tris buffer (pH 8.0), at 30°C for 30 min. By the addition of an equal volume of ethanol, the reaction will be terminated and the solution analysis for celastrol will be done with ultra-performance liquid chromatography (UPLC).

UPLC and HPLC Analysis. 1 mg of the solution will be vortexed in deionized-distilled water at 37°C. Then, the mixture will be centrifuged at 10,000 x g and will be filtered through a nylon membrane at 25°C. The filtrate will be mixed with an equal volume of methanol and UPLC analysis will be conducted. The UPLC system will be equipped with an analytic C-18 reversed-phase column. The conditions of the system will be a gradient elution using water containing %1 acetic acid, and methanol with a linear gradient for 11 minutes with %50-80 methanol and then 2 minutes with %80-100 methanol, an injection volume of 0.2 µL. The analyte detection will be done with optical absorbance at 430 nm. After filtration through a nylon membrane, the mixture will be injected into a preparative C-18 reversed-phase column HPLC system. The operational conditions will be the same as those in UPLC. The elution corresponding to the peak of the metabolite in UPLC will be collected, concentrated and then lyophilized.

Determination of Solubility of Celastrol glycosides. To examine the aqueous solubility of celastrol and its glucoside derivative, 1 mg of each compound will be vortexed in distilled water for 4 hours at 37 °C. The resulting mixture will be centrifuged at 10,000 × g for 30 minutes and will be filtered using a nylon membrane at 25 °C. After filtration, the solution will be mixed with methanol and will be analysed by UPLC. The concentration values of the tested compounds will be determined according to the peaks using the calibration curves prepared as a result of the UPLC analysis of the samples dissolved in DMSO.

Determination of Toxicity with DPPH Free Radical Scavenging Activity. The antioxidant activity of the modified celastrol will be determined using the DPPH free radical scavenging assay system. The sample dissolved in DMSO will be added to the DPPH solution and will be incubated for 30 minutes. As a positive antioxidant control, ascorbic acid which was dissolved in DMSO will be used. After 30 minutes of reaction, the absorbance of the reaction mixture and positive control will be read at 517 nm with a UV-VIS spectrophotometer.

Toxicological Analysis of Modified Celastrol onto Cells by MTS Toxicity Assay. For the toxicology analysis, celastrol samples in different concentrations will be tested on cells *in vitro*. In the first step, the assay will be conducted with healthy cell lines, mouse embryonic fibroblast (3T3) and human fetal osteoblastic cells (hFOB). The needed amount of cell suspension and MTS media (starvation media and complete media) will be found through cell counting with a hemocytometer. Then, a necessary amount of cell suspension and MTS media will be prepared for 4 different celastrol conditions (0.2-1-2-10 µl/mL). After 24 hours of incubation for cell growth, celastrol prepared in different concentrations will be added to cell cultures. Then, cultures will be incubated for 24h, 48h, and 72h. After these incubations, MTS solutions containing PES will be added to all samples and will be incubated for 1-4 hours at 37°C. Absorbance will be measured at 490 nm. All these procedures will also be applied to cancer cell lines which will be human osteosarcoma (SaOS-2) and human breast cancer cells (MCF-7).

RESULTS

After the optimization of the Celastrol, molecular docking of glycosyltransferase enzyme and celastrol will be performed, and then by looking at the free energy values coming from the docking, the optimum pose for the celastrol will be given to the molecular dynamics simulations. Since docking is scholastic and not time-dependent, in order to see changes through time at the atomic level, Molecular Dynamic Simulations are necessary. Both monomer and dimer structures will be simulated. As a result, the stability of the celastrol during the simulation, the interaction between drug and sugar moiety, and the differences between monomer and dimer structure throughout the simulation will be analyzed and by using MM/PBSA method, binding free energies in the presence of a drug and the absence of drug will be calculated and compared to be able to quantitatively analyze the bindings. Finally, to be able to get a more reliable free energy calculation and verify our results, the Thermodynamic Integration technique will be used.

First, single colonies that are expected to grow in kanamycin-selective agar media will be used to produce a high amount of glycosyltransferase protein in broth media. In order to purify the glycosyltransferase enzyme, the His-Tag purification procedure with Ni+2 chelate affinity column will be followed, since the expression vector also contains the polyhistidine (6xHis) tag. The SDS-PAGE result for the glycosyltransferase enzyme is expected to indicate that the culture selection from selective media, the expression of the transformed vector and the His-Tag purification steps are completed successfully.

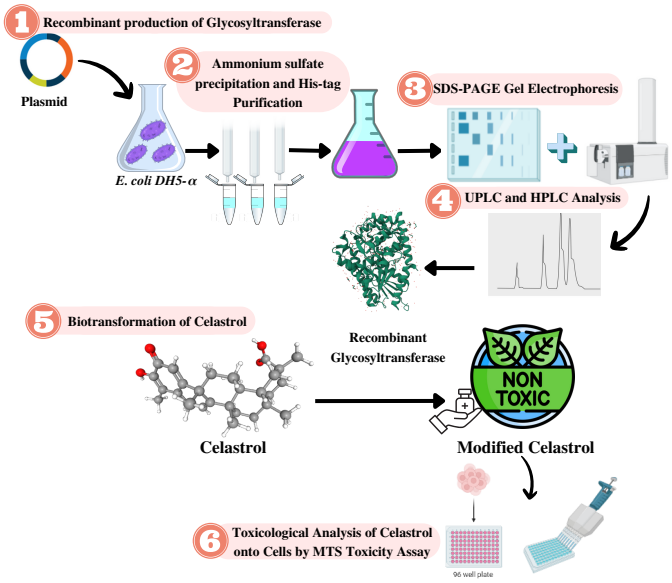

Figure 1. Schematic representation of methods that used in this experiment

The BenchMark™ Protein Ladder will demonstrate the purified protein migrated as two bands in 50 kDa, proving the total 100.79 kDa dimeric Glycosyltransferase existence.

The enzymatic activity of recombinant glycosyltransferase will be measured by Glycosyltransferase Activity Kit from R&D systems. It is expected that the activity of the glycosyltransferase enzyme is plotted against enzyme input. As a result, the specific activity of recombinant GT must be closely determined as 1806 pmol/min/µg.

After the validation of glycosyltransferase enzyme activity is completed, the biotransformation of celastrol with this enzyme will be performed. After the biotransformation process, the product yields of the modified celastrol and wild-type celastrol will be calculated by UPLC. The yields of modified celastrol are expected to be high (~42%). The system was set up for a gradient elution using water that contained acetic acid and a linear gradient of methanol for 13 minutes.

The analyte detection is expected to be observed with two peaks with an optical absorbance of 430 nm. Furthermore, the results are expected to be supported by the HPLC analysis. The MTS assay will determine the cytotoxicity of Celastrol. In order to investigate the effects of modified celastrol on healthy cells, two cell lines 3T3 and hFOB will be treated both with four different wild-type celastrol and modified celastrol concentrations for 24h, 48h, and 72h, then MTS assay is used to measure the cell viability. As a result, it is expected to increase cell viability by at least 50% in both cell lines. Depending on the eligible dosage of modified celastrol amount from healthy cells, the MTS assay will be performed on cancer cell lines (SaOS-2 and MCF-7) to assess the effect of modified celastrol on cancer cells. In addition to the MTS assay, DPPH free radical scavenging assay will be performed to support the results of the MTS assay.

Until these steps, glycosyltransferase enzymes have been produced, celastrol molecules have been modified, and then the toxicity of modified molecules has been tried on both healthy and cancer cells. In the final step, the chemical properties of the modified celastrol will be examined according to stability, low toxicity and increased solubility

CONCLUSION

In this project, it is aimed to decrease the toxicity of celastrol, which carries the potential to be used in the medical and pharmaceutical field, through biotransformation via synthetic biology approaches. Throughout this project proposal, the designed experimental parts, and the importance of this project were described in detail.

As a first step of our hypothesis, we will demonstrate this model in cancer and healthy cell lines to assess the reduced toxicity. In this way, it is expected to induce apoptosis in most of the tumour cells without any damage to healthy cells. Among the studies conducted to biotransform celastrol in the literature, our work is the first experimental design which uses this specific glycosyltransferase enzyme to achieve modification of that molecule. This project has the potential to cure many diseases in addition to cancer by medicating the biotransformed celastrol, which includes high mortality rates and affects billions of people across the world. With the properties of modified celastrol, it can also be used in the treatment of cardiovascular diseases, obesity, viral infections, Parkinson's Disease, Alzheimer's Disease and many others.

The purpose of this project is to use biotransformed celastrol as an accessible and affordable medicine for humanity. More research is needed to confirm the findings and to determine the appropriate dosages and long-term safety of celastrol. Even in further studies, celastrol carries the potential to be inducted in drug form. The resulting novel combination may serve as a potential candidate in future clinical applications.

BIOSAFETY

In this project, all biosafety and blacklist criteria defined by GOGEC were taken into account and complied with. All parts of the experiments were designed according to these criteria. The organism that is selected as the host is a biosafety level 1 organism. All of the chemicals were chosen to be non-toxic chemicals.

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