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# CRISPR-Mediated Transcriptional Activation of Squalene Synthase to Increase the Biosynthesis of Anticancer Metabolite Inotodiol in *Inonotus obliquus*: A Research Protocol

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**Abstract**
Chaga (*Inonotus obliquus*) is a parasitic fungus of birch trees used in the traditional medicine of Russia, China, and other Eurasian countries. The secondary metabolites produced by *I. obliquus* are known to possess anticancer, anti-inflammatory, antiviral, antioxidant, and hypoglycemic activity, while posing no known adverse effects. The therapeutic properties of *I. obliquus* are thus of particular medical interest. Several studies have demonstrated that the triterpenoids produced by *I. obliquus* provide the anticancer effects. Specifically, the triterpenoid inotodiol has demonstrated promising antitumor effects in human cervical cancer HeLa cells. Unfortunately, the limited natural abundance of *I. obliquus* impedes use of this fungus as a source of inotodiol for clinical applications. Furthermore, attempts to culture *I. obliquus* in a laboratory setting are limited by the low expression of biosynthetic gene clusters. While chemical syntheses of inotodiol avoid these challenges, they are hindered by low yield, cost, and time. To address these challenges, we propose using CRISPR-mediated transcriptional activation (CRISPRa) to boost the expression of the enzyme (squalene synthase (SQS)) that mediates the biosynthesis of inotodiol in *I. obliquus*, thereby increasing production of the therapeutic metabolite. The level of inotodiol production achieved by cultured *I. obliquus* cells treated with endonuclease deficient Cas9 fused to transcriptional activator domains (dCas9-VPR) will be compared using three test groups with single guide RNAs (sgRNAs) varying in their distance upstream from the SQS gene, and a control group with no sgRNA. To evaluate the proposed CRISPRa system, transcriptomic, proteomic, and metabolomic analyses will be implemented. To our knowledge, CRISPRa methodology has not yet been used to improve the yield of *I. obliquus* metabolites.

**Keywords:** *Inonotus obliquus*; inotodiol; anticancer; secondary metabolites; biosynthesis; BGCs; CRISPRa; dCas9-VPR; sgRNA; multiomics

## Introduction

Fungi are an important source of bioactive secondary metabolites (SMs) for drug discovery. SMs are small molecules synthesized within an organism that provide a selective advantage, but are not required for survival [1]. The fungus *Inonotus obliquus*, popularly referred to as chaga, is known to produce many SMs with therapeutic properties. *I. obliquus* is found in several northern Eurasian countries, where it is used in traditional medicinal practices. The most clinically important SM produced by *I. obliquus* is inotodiol, a triterpenoid. Triterpenoids are a group of cyclic compounds consisting of a six-isoprene unit structure, with many having alcohol, aldehyde, or carboxylic acid functional groups [2]. In fungi, triterpenoids are produced via biosynthesis from squalene, a thirty-carbon, acyclic, triterpene hydrocarbon [2]; interestingly, triterpenoids are the largest group of phytochemicals—over 20,000 naturally occurring triterpenoids have been characterized [3]. Despite established use in traditional Eurasian medicine, triterpenoids have only recently gained interest in modern medicine due to their diverse pharmacological activities and low toxicity [3]. Importantly, several triterpenoids have exhibited cancer-selective toxicity and antitumour efficacy in various cancer cell lines and animal models [3]. With a structure similar to that of cholesterol and lanosterol, the tetracyclic triterpenoid inotodiol is unique in that it possesses an additional hydroxyl group at carbon 22, which is believed to confer a variety of therapeutic properties [4]. Moreover, inotodiol is only found in *I. obliquus*, where it is produced biosynthetically [4]. Of particular interest is that, in study, inotodiol has demonstrated potent antitumor effects against human cervical cancer models [5, 6]. Specifically, it has been reported that in human

cervical cancer HeLa cells, inotodiol inhibits cell migration, invasion, and proliferation, even inducing apoptosis via a p53-dependent pathway [5, 6]. The mechanism of such anticancer effects includes downregulation of MMP-2 and MMP-9 (migration and invasion) and Bcl-2 (antiapoptosis), as well as upregulation of PARP, caspase 3, Bax, etc. (proapoptosis) [5, 6]. Importantly, inotodiol-based drugs can be engineered to selectively target cancer; a recent in vitro study demonstrated that folate-targeting inotodiol liposomes are cytotoxic to cancer cells while harming few healthy cells [7]. Such selective toxicity is due to the high expression of folate receptors in carcinomas (e.g, cervical cancer), highlighting the potential of inotodiol as a targeted cancer therapeutic. Encouragingly, several clinical experiments involving *I. obliquus* SMs have reported additional therapeutic activities (e.g., anti-inflammatory, antiviral, antioxidant, and hypoglycemic) without adverse effects [8]. Specifically, the safety profile of inotodiol has been established through several clinical experiments; in one such study, administration of crude and pure inotodiol extract in mouse models of cancer at 30 mg/kg and 20 mg/kg, respectively, did not produce hepatoxicity or inhibition of cytochromes [9]. Furthermore, inotodiol is not known to alter any biochemical or hematological parameters or lipid metabolism, and it has low blood-brain permeability [9]. While the clinical efficacy of inotodiol (and other *I. obliquus* SMs) has been demonstrated, there are limitations to its isolation. *I. obliquus* is found in cold environments where it grows very slowly, limiting its utility (it has been reported that a practically sized *I. obliquus* conk requires up to 15 years of growth) [10, 11].Moreover, in fungi, the genes responsible for biosynthesis are clustered and have complex regulatory mechanisms that are often only active in natural conditions [12]. Consequently, attempts to isolate SMs via cell culture result in only modest yields due to the low expression of biosynthetic gene clusters (BGCs) in typical laboratory conditions [11]. While chemical synthesis of inotodiol avoids these challenges, it is significantly hindered by low yield (loss of absolute configuration), cost (reagents, equipment, labor), and time (multi-step synthesis) [13].

In *I. obliquus*, inotodiol is synthesized through the fungal mevalonate metabolic pathway [14]. This biosynthetic pathway has three parts: precursor pathway, sesquiterpenoid pathway, and triterpenoid pathway (Figure 1) [14]. In this protocol, we are primarily interested in the sesquiterpenoid pathway. In the sesquiterpenoid pathway, SQS catalyzes a condensation reaction in which two farnesyl diphosphate (FPP) molecules form a squalene molecule [14]. This reaction is critical as squalene is the first committed precursor for the biosynthesis of inotodiol. Furthermore, *I. obliquus* transcriptome studies have shown that in laboratory conditions SQS is among the least expressed enzymes in this pathway, probably due to a complex promoter [14]. It has also been shown that downstream enzymes such as squalene epoxidase (SQE) are upregulated in the presence of increased triterpenoid precursor (e.g., squalene), resulting in greater flux through the triterpenoid pathway and thus increased inotodiol production [14]. These findings, along with catalyzing the formation of the first committed precursor, suggest an essential role for SQS in inotodiol biosynthesis. Thus, to overcome the limitation of laboratory silent BGCs, SQS is an excellent target for artificially increasing gene expression.

To boost the expression of SQS, and thereby increase the biosynthesis of inotodiol, we propose using clustered regularly interspaced short palindromic repeats (CRISPR)-mediated transcriptional activation (CRISPRa). Traditionally, CRISPR is used as a genome editing tool in which a CRISPR associated protein (Cas) performs targeted DNA cleavage via its endonuclease domains; a customizable single guide RNA (sgRNA) is used to direct Cas to a target locus where Cas-induced double-stranded (CRISPR/Cas nucleases) or single-stranded (base and prime editors) breaks enable precision genome editing. However, in CRISPRa, there is no DNA cleavage or genome editing; rather, transcriptional activation of a target gene is achieved via a mutant Cas9 lacking endonuclease activity (dCas9) fused to transcriptional activators, complexed with a sgRNA [15]. As alluded to above, the sgRNA is a short sequence that is complementary to the target sequence, and it is used to physically bring Cas to the target genomic site upon hybridization with it. In CRISPRa, the sgRNA is designed to be complementary to a sequence upstream of the target gene's transcriptional start site (TSS); specifically, the sgRNA binds directly upstream of a Cas-specific protospacer adjacent motif (PAM)—Cas9 recognizes the PAM sequence NGG (where "N" is any nucleotide base). In CRISPRa systems, fusion of transcriptional activator domains to dCas9 enables transcriptional activation of sgRNA-directed genomic targets. In one such system, validated transcription factors are fused to form a tripartite activator: four copies of herpes simplex viral protein 16 (VP64), the 65 kDa subunit of nuclear factor kappa B (p65), and Epstein-Barr virus R transactivator (RTA) are fused to the C-terminus of dCas9, giving dCas9-VP64-p65-RTA (VPR) [16]. Such a combination of transcriptional activators results in recruitment of various transcriptional machinery, including transcription factors and chromatin remodelers (e.g., AP-1, ATF/CREB, SP1, PC4, CBP/p300, SWI/SNF complex) [17]. In study, dCas9-VPR has demonstrated robust transcriptional activation, producing gene expression many-fold higher than background and in one study, 320-fold higher than other CRISPRa systems, such as dCas9-VP64 [18]. In essence, the customized sgRNA directs dCas9-VPR to the target gene, where it binds within the promoter region, inducing gene expression via recruitment of transcription factors, chromatin remodelers, and ultimately RNA polymerases by its fused activators [16, 17].

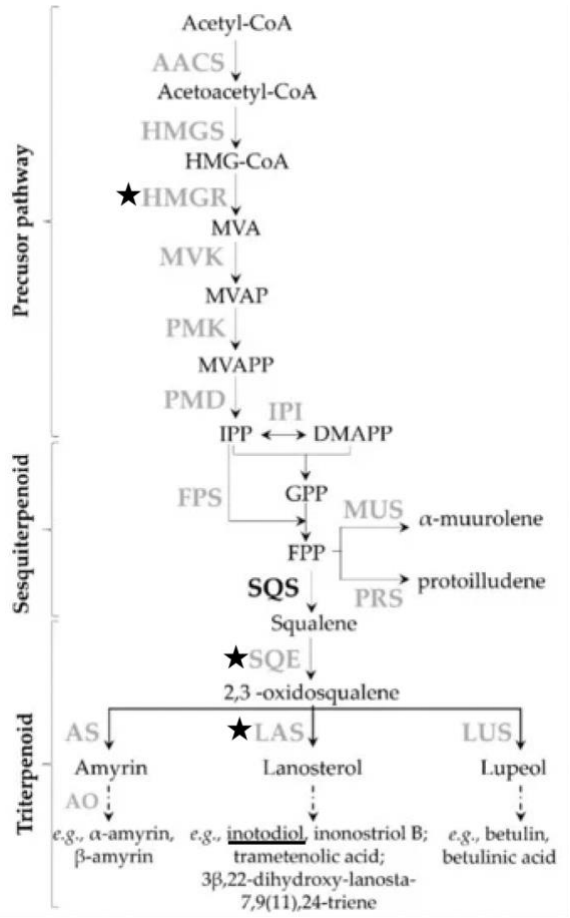


**Figure 1.** Enzymes involved in the biosynthesis of inotodiol. Squalene synthase (SQS) is currently the only sequenced gene. Future CRISPRa targets are labelled with a star (see Conclusion). Adapted from Fradj et al. [14].

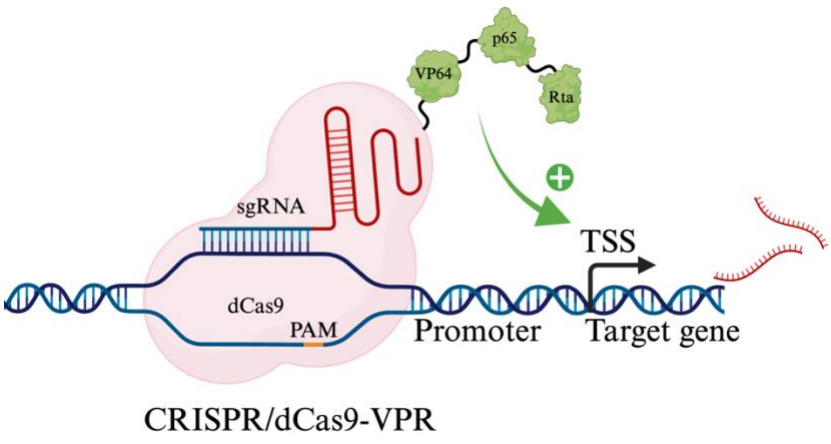


**Figure 2.** The CRISPR/dCas9-VPR system consists of a mutant Cas9 lacking endonuclease activity (dCas9) fused to transcriptional activators (VPR) complexed with a sgRNA. Created with BioRender.com.

Here, we propose using the pre-established CRISPR/dCas9-VPR system to increase the expression of the SQS gene. In previous studies, this CRISPRa system has been successfully implemented to activate silent BGCs in

other fungal species [1, 15]. Therefore, we hypothesize that a CRISPR/dCas9-VPR system will boost the expression of the SQS gene in cultured *I. obliquus* cells, increasing the yield of the anticancer SM inotodiol.

**Methods**

The following will be purchased from appropriate providers: three differently customized sgRNAs (Table 1) and DNA sequences for the dCas9-VPR protein complex. These will be integrated into single-vector plasmids and subsequently amplified using *E. coli* bacteria; antibiotic resistance genes (AmpR) will also be integrated into the plasmids, allowing for positive selection via antibiotic treatment. *I. obliquus* samples and all reagents necessary for cell culture and various analytic techniques will also be purchased. Samples will be cultured for 8 days in Petri dishes, each starting from a single pellet in a liquid malt broth medium at 28 °C and pH 6.2 [14, 19]. Cultures will then be treated with antibiotic (ampicillin) to select for the desired plasmids. Following the growth period, plasmid extraction from *E. coli* will be performed; plasmids containing only the dCas9-VPR complex will be added to the control group, and plasmids containing dCas9-VPR complexes and one out of three sgRNAs will be added to the test groups. The sgRNA sequences for test groups one, two, and three will be designed to bind to the following sites upstream of the TSS, respectively: -97 bp, -255 bp, and -400 bp.

**Table 1.** sgRNAs Designed to Bind at Varying Distances Upstream of the SQS Gene's TSS

| Test group | Number of bp upstream of TSS | Sequence (5'-3') |
|---|---|---|
| 1 | 97 | GAATAGTCTGCGTCAGAGGT |
| 2 | 255 | CAAAGGTTCCCGTGTGAAGT |
| 3 | 400 | CGTTTGGAGGGGCAGCAAGC |

To efficiently deliver plasmids to *I. obliquus* cells, the commonly used polyethylene glycol (PEG)-mediated transformation will be implemented as it has proved successful in many fungal species [22]. To begin this procedure, transformable *I. obliquus* protoplasts will be prepared by applying the following cell wall degrading enzymes to cultured cells: cellulase, 1,3-glucanase, chitinase, and driselase [22]. Plasmids will be pipetted into centrifuge tubes in a 10:1 ratio to protoplasts, as determined by Mózsik et al [1]. A high concentration of PEG in the presence of calcium ions will be added to facilitate uptake of plasmid DNA (pDNA) [22]. These mixtures will be centrifuged to pellet the protoplasts, allowing for efficient isolation [22]. Fungal pellets will be added to regeneration media and incubated for 6 days to allow plasmid transformation and accommodate CRISPR-associated cellular processes [1]. Subsequently, transcriptomic, proteomic, and metabolomic analyses will be performed to assess the performed experiments. Each culture will be aliquoted 3 times daily, for 5 days. Every day, one aliquot from each group will undergo reverse transcription-quantitative polymerase chain reaction (RT-qPCR) to quantify mRNA transcripts of the SQS gene (Table 2).

**Table 2.** RT-qPCR Primers for Amplification of SQS Gene Transcripts

| Primer | Sequence (5'-3') |
|---|---|
| Forward | ATGGGTGCGACAGACATGATG |
| Reverse | CTACGAGAAATACTTCATAAC |

Another aliquot from each group will undergo Western blotting to detect and measure the relative quantity of translated SQS enzymes. The final aliquot from each test group will undergo high-speed counter-current chromatography (HSCCC), evaporative light-scattering detection (ELSD), and tandem mass spectrometry (MS/MS) to quantify synthesized inotodiol [23]. The three quantitative techniques will also each be performed on a control aliquot, allowing for direct comparison.

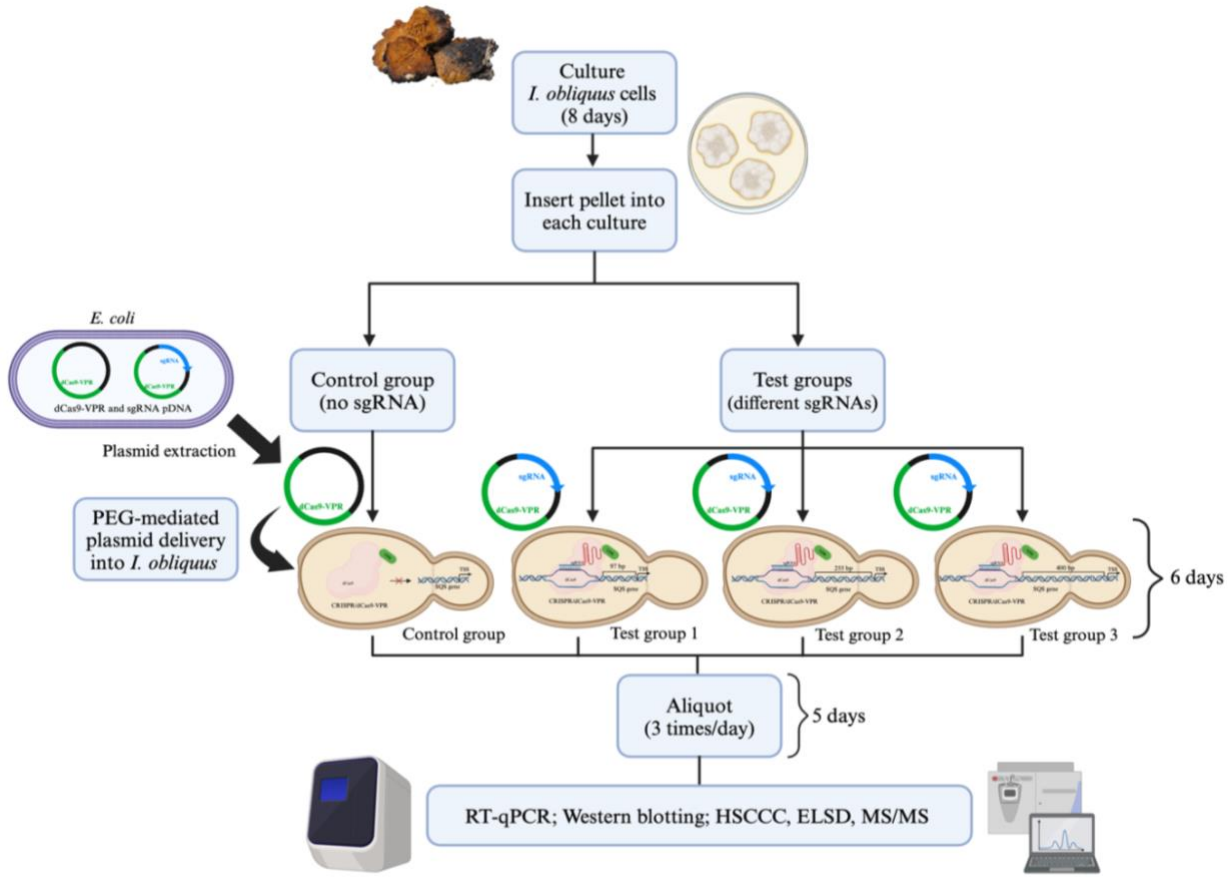


**Figure 3.** Methods Summary Highlighting the Culturing of Fungal Cells, Experimental Design, and Quantitative Techniques. Created with BioRender.com.

**Results**

There are no results to report as the outlined experiments have not been performed. However, if performed, it is expected that the three test groups will show significantly increased levels of SQS transcript, SQS enzyme, and inotodiol in comparison to the control group. The control is not expected to show significant changes at any level of expression as there is no sgRNA to direct dCas9-VPR to the SQS gene.

**Discussion**

Implementation of RT-qPCR; Western blotting; and HSCCC, ELSD, and MS/MS in control group aliquots will allow for approximation of background levels of SQS transcript, SQS enzyme, and inotodiol, respectively. From this data, we would expect the three test groups to exhibit increased expression of the SQS gene and thus increased levels of SQS enzyme, leading to greater flux through the sesquiterpenoid pathway and ultimately, increased inotodiol biosynthesis. Variation in the upstream distance (from the TSS) of the test groups' sgRNA binding site will provide insight into the promoter region of the SQS gene, informing future experiments with optimally binding sgRNAs. While all three test groups are predicted to increase inotodiol production in comparison to the control, considerable variation is expected and will be key for informing the development of future CRISPRa systems targeted to SQS. Rigorous statistical analyses will be performed to compare collected data between test and control groups, ensuring that any observed changes are not simply due to random variation in background expression levels ($p < 0.05$). Specifically, Kruskal-Wallis and post-hoc Dunn's test will allow for direct comparison between aliquot groups for each of the five days of data collection.

Furthermore, transcriptomic, proteomic, and metabolomic analyses will allow us to scrutinize the proposed system at all levels of expression to identify any limitations in the current model. For instance, SQS's substrate, FPP, is also a substrate for muurolene synthase (MUS) and protoilludene synthase (PRS), preventing complete flux through SQS even with increased expression. Additionally, downstream of SQS, the triterpenoid pathway divides into three pathways as 2,3-oxidosqualene (formed from squalene via squalene epoxidase (SQE)) is also a substrate of amyrin synthase (AS) and lupeol synthase (LUS) in addition to lanosterol synthase (LAS), the enzyme that leads to inotodiol production. Altogether, the complexity of

the pathways involved in inotodiol biosynthesis may limit the efficacy of CRISPRa of SQS, despite its critical role. Another foreseeable limitation is in the timing of the aliquots used to collect data. It is unclear as to the timeframe between plasmid uptake, the induction of SQS expression, and the CRISPR-associated processes in between (i.e., assembly of dCas9-VPR/sgRNA effector complex, target hybridization, gene expression, complex degradation); a latency period of one day may not be optimal. Thus, future studies should involve aliquot and analysis after a shorter period (e.g., within hours of PEG-mediated transformation of plasmids). While other CRISPR systems—particularly those involving DNA cleavage—come with the limitation of off-target effects, this is not the case with CRISPRa as off-target sequences must not only match the target sequence but also be part of a promoter region [24].

## Conclusions

To overcome the challenges in isolating inotodiol from *I. obliquus* (i.e., cold environment, silent BGCs, poor chemical synthesis), a novel CRISPR/dCas9-VPR system for boosting the expression of the SQS gene is proposed. The predicted increased yields of inotodiol (and other clinically relevant SMs) will facilitate drug discovery. Furthermore, increased inotodiol availability will reduce research and development costs, ultimately reducing future market prices of drugs based on inotodiol. Specifically, inotodiol-based drugs will offer a promising, novel therapeutic approach to human cancers, particularly cervical cancer. Considering the adverse effects and high costs associated with current cancer therapies (e.g., chemotherapy), the safety and affordability associated with inotodiol further supports its clinical relevance and the dire need to address isolation challenges.

Currently, SQS is the only gene that has been isolated and sequenced from the fungal mevalonate metabolic pathway. Although SQS has an important regulatory role, future research should be directed towards sequencing downstream enzymes squalene epoxidase (SQE) and lanosterol synthase (LAS) as they could be targets for a similar CRISPRa system (see Figure 1). Moreover, a multiplex CRISPRa system targeted to all three enzymes could increase specificity and substantially boost inotodiol production. Another future target for gene sequencing is 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR), the rate-limiting enzyme in terpenoid biosynthesis. Boosting HMGR expression would increase the formation of key intermediate mevalonate, resulting in downstream increases in inotodiol [14]. Finally, to simplify experimental procedures and create opportunity for more continuous inotodiol isolation, *I. obliquus* cell lines that stably express dCas9-VPR should be generated. Such transgenic cell lines would only require plasmid delivery of sgRNAs, making for a more robust CRISPRa system that would likely result in greater yields of inotodiol over time [24].

## List of Abbreviations Used

AmpR: ampicillin resistance gene
AP-1: activator protein 1
AS: amyrin synthase
ATF/CREB: activating transcription factor/cyclic AMP response element binding protein
Bax: bcl-2-associated X-protein
Bcl-2: B-cell lymphoma 2
BGCs: biosynthetic gene clusters
Cas: CRISPR-associated protein
Cas9: CRISPR-associated protein 9
CBP/p300: cyclic AMP response element-binding protein/histone acetyltransferase protein 300
CRISPR: clustered regularly interspaced short palindromic repeats
CRISPRa: CRISPR-mediated transcriptional activation
dCas9: endonuclease deficient Cas9
dCas9-VPR: VPR fused to dCas9
DNA: deoxyribonucleic acid
ELSD: evaporative light-scattering detection
FPP: farnesyl diphosphate
HMGR: 3-hydroxy-3-methylglutaryl-coenzyme A reductase
HSCCC: high-speed counter-current chromatography
LUS: lupeol synthase
MMP-2: matrix metalloproteinase-2
MMP-9: matrix metalloproteinase-9
MUS: muurolene synthase
mRNA: messenger RNA
MS/MS: tandem mass spectrometry
PAM: protospacer adjacent motif
PARP: poly (ADP-ribose) polymerase
pDNA: plasmid DNA
PEG: polyethylene glycol
p65: 65 kDa subunit of nuclear factor kappa B
PC4: positive cofactor 4
PRS: protoilludene synthase
RNA: ribonucleic acid
RTA: replication and transcription activator
RT-qPCR: reverse transcription-quantitative polymerase chain reaction
sgRNA: single guide RNA
SM: secondary metabolite
SP1: specificity protein 1
SQE: squalene epoxidase
SQS: squalene synthase
SWI/SNF: SWItch/sucrose non-fermentable
TSS: transcriptional start site
VP16: herpes simplex viral protein 16
VP64: four copies of VP16
VPR: VP64-p65-Rta

**Conflicts of Interest**
The authors declare that they have no conflicts of interest.

**Ethics Approval and/or Participant Consent**
This study did not require ethics board approval or participant consent as it is a theoretical research protocol in which no experiments were conducted.

**Authors' Contributions**
AGJ: conducted literature review, generated and interpreted key concepts, made substantial contributions to the design and writing of the research protocol, drafted the manuscript, and gave final approval of the version to be published.
DTR: conducted literature review, generated and interpreted key concepts, made substantial contributions to the design and writing of the research protocol, drafted the manuscript, and gave final approval of the version to be published.

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**References**
[1] Mózsik L, Hoekzema M, de Kok NAW, Bovenberg RAL, Nygård Y, Driessen AJM. CRISPR-based transcriptional activation tool for silent genes in filamentous fungi. Sci Rep 2021;11:1118. https://doi.org/10.1038/s41598-020-80864-3
[2] Ludwiczuk A, Skalicka-Woźniak K, Georgiev MI. Chapter 11 - Terpenoids. In: Badal S, Delgoda R, editors. Pharmacognosy, Boston: Academic Press; 2017, p. 233–66. https://doi.org/10.1016/B978-0-12-802104-0.00011-1
[3] Bishayee A, Ahmed S, Brankov N, Perloff M. Triterpenoids as potential agents for the chemoprevention and therapy of breast cancer. Front Biosci J Virtual Libr 2011;16:980–96. https://doi.org/10.2741%2F3730
[4] Nguyen PC, Nguyen MTT, Truong BT, Kim D-R, Shin S, Kim J-E, et al. Isolation, Physicochemical Characterization, and Biological Properties of Inotodiol, the Potent Pharmaceutical Oxysterol from Chaga Mushroom. Antioxidants 2023;12:447. https://doi.org/10.3390/antiox12020447
[5] Zhao L-W, Zhong X-H, Yang S-Y, Zhang Y-Z, Yang N-J. Inotodiol inhabits proliferation and induces apoptosis through modulating expression of cyclinE, p27, bcl-2, and bax in human cervical cancer HeLa cells. Asian Pac J Cancer Prev APJCP 2014;15:3195–9. https://doi.org/10.7314/apjcp.2014.15.7.3195
[6] Zhang S-D, Yu L, Wang P, Kou P, Li J, Wang L-T, et al. Inotodiol inhibits cells migration and invasion and induces apoptosis via p53-dependent pathway in HeLa cells. Phytomedicine Int J Phytother Phytopharm 2019;60:152957. https://doi.org/10.1016/j.phymed.2019.152957
[7] Lu Y, Gao X, Chen Z, Xue Z, Jia Y, Li N, et al. Characterization and Anticancer Effects of Folate Targeted Inotodiol Liposome From Inonotus Obliquus (Chaga Mushroom 2021. https://doi.org/10.21203/rs.3.rs-242884/v1
[8] Lu Y, Jia Y, Xue Z, Li N, Liu J, Chen H. Recent Developments in Inonotus obliquus (Chaga mushroom) Polysaccharides: Isolation, Structural Characteristics, Biological Activities and Application. Polymers 2021;13:1441. https://doi.org/10.3390/polym13091441
[9] Nguyen TMN, Ban S-Y, Park K-B, Lee C-K, Lee S-W, Lee Y-J, et al. Evaluation of Toxicity and Efficacy of Inotodiol as an Anti-Inflammatory Agent Using Animal Model. Molecules 2022;27:4704. https://doi.org/10.3390/molecules27154704
[10] Duan Y, Han H, Qi J, Gao J, Xu Z, Wang P, et al. Genome sequencing of Inonotus obliquus reveals insights into candidate genes involved in secondary metabolite biosynthesis. BMC Genomics 2022;23:314. https://doi.org/10.1186/s12864-022-08511-x
[11] Zheng W, Miao K, Liu Y, Zhao Y, Zhang M, Pan S, et al. Chemical diversity of biologically active metabolites in the sclerotia of Inonotus obliquus and submerged culture strategies for up-regulating their production. Appl Microbiol Biotechnol 2010;87:1237–54. https://doi.org/10.1007/s00253-010-2682-4
[12] Hoskisson PA, Seipke RF. Cryptic or Silent? The Known Unknowns, Unknown Knowns, and Unknown Unknowns of Secondary Metabolism. mBio 2020;11:10.1128/mbio.02642-20. https://doi.org/10.1128/mbio.02642-20
[13] Kim S, Go E, Song M, Park G, Lee E, Bae S, et al. A novel method for producing inotodiol [Internet]. 2022 [cited 2024 Aug 9]. Available from: https://patents.google.com/patent/KR20220011502A/en. Korean

[14] Fradj N, Gonçalves Dos Santos KC, de Montigny N, Awwad F, Boumghar Y, Germain H, et al. RNA-Seq de Novo Assembly and Differential Transcriptome Analysis of Chaga (Inonotus obliquus) Cultured with Different Betulin Sources and the Regulation of Genes Involved in Terpenoid Biosynthesis. Int J Mol Sci 2019;20:4334. https://doi.org/10.3390/ijms20184334

[15] Roux I, Woodcraft C, Hu J, Wolters R, Gilchrist CLM, Chooi Y-H. CRISPR-Mediated Activation of Biosynthetic Gene Clusters for Bioactive Molecule Discovery in Filamentous Fungi. ACS Synth Biol 2020;9:1843–54. https://doi.org/10.1021/acssynbio.0c00197

[16] Liu S, Striebel J, Pasquini G, Ng AHM, Khoshakhlagh P, Church GM, et al. Neuronal Cell-type Engineering by Transcriptional Activation. Front Genome Ed 2021;3. https://doi.org/10.3389/fgeed.2021.715697

[17] Konermann S, Brigham MD, Trevino AE, Joung J, Abudayyeh OO, Barcena C, et al. Genome-scale transcriptional activation by an engineered CRISPR-Cas9 complex. Nature 2015;517:583–8. https://doi.org/10.1038/nature14136

[18] Chavez A, Scheiman J, Vora S, Pruitt BW, Tuttle M, Iyer E, et al. Highly-efficient Cas9-mediated transcriptional programming. Nat Methods 2015;12:326–8. https://doi.org/10.1038/nmeth.3312

[19] Reeve MA, Bachmann D. A method for filamentous fungal growth and sample preparation aimed at more consistent MALDI-TOF MS spectra despite variations in growth rates and/or incubation times. Biol Methods Protoc 2019;4:bpz003. https://doi.org/10.1093/biomethods/bpz003

[20] Zheng F, Liu N, Che Y, Zhang L, Shao L, Zhu J, et al. Cloning, expression and characterization of squalene synthase from Inonotus obliquus. Genes Genomics 2013;35:631–9. https://doi.org/10.1007/s13258-013-0113-5

[21] Hou H. ASM3216429v1 [Internet]. Inonotus obliquus genome assembly. School of Bioscience and Technology; 2023 [cited 2024 Jun 1]. Available from: https://www.ncbi.nlm.nih.gov/datasets/genome/GCA_032164295.1/

[22] Liu Z, Friesen TL. Polyethylene glycol (PEG)-mediated transformation in filamentous fungal pathogens. Methods Mol Biol Clifton NJ 2012;835:365–75. https://doi.org/10.1007/978-1-61779-501-5_21

[23] Du D, Zhu F, Chen X, Ju X, Feng Y, Qi L-W, et al. Rapid isolation and purification of inotodiol and trametenolic acid from Inonotus obliquus by high-speed counter-current chromatography with evapo rative light scatting detection. Phytochem Anal PCA 2011;22:419–23. https://doi.org/10.1002/pca.1297

[24] Becirovic E. Maybe you can turn me on: CRISPRa-based strategies for therapeutic applications. Cell Mol Life Sci CMLS 2022;79:130. https://doi.org/10.1007/s00018-022-04175-8

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