3-25-2021

Identification of Human Topoisomerase I Poison and Catalytic Inhibitors

Christian Madeira
Florida International University, cmade005@fiu.edu

FLORIDA INTERNATIONAL UNIVERSITY

Miami, Florida

IDENTIFICATION OF HUMAN TOPOISOMERASE I POISON AND CATALYTIC INHIBITORS

A thesis submitted in partial fulfillment of

the requirements for the degree of

MASTER OF SCIENCE

in

CHEMISTRY

by

Christian Madeira

2021

To: Dean Michael R. Heithaus

College of Arts, Sciences and Education

This thesis, written by Christian Madeira, and entitled Identification of Human Topoisomerase I Poison and Catalytic Inhibitors, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this thesis and recommend that it be approved.

_____
Yuan Liu

_____
Jaroslava Miksovska

_____
Yuk-Ching Tse-Dinh, Major Professor

Date of Defense: March 25, 2021

The thesis of Christian Madeira is approved.

_____
Dean Michael R. Heithaus
College of Arts, Sciences and Education

_____
Andrés G. Gil
Vice President for Research and Economic Development
and Dean of the University Graduate School

Florida International University, 2021

DEDICATION

I dedicate this thesis to my immediate and extended family. Without their patience, understanding, and most of all, love, the completion of this work would not have been possible. I would also like to give special consideration to my loving cousin Nesha who unfortunately passed away after her heroic battle with acute myeloid lymphoma. I have promised her that I will better myself and to continue to overcome life's major obstacles.

To Nesha,

"Even at your worst moments, you had faith and hope in god that he'll steer us in the right direction. I hope I can continue to make you proud."

Most importantly, I would be nowhere without my grandmother Faneera, who passed away on March 11, 2021 reassured me that if I continue being ambitious and handle failures constructively, I will be unable to be defeated.

To Grandma,

"You made sure your family was taken care of at the expense of your health. If anything, Not seeing you here anymore makes me want to work twice as more."

ACKNOWLEDGMENTS

All members from each facet of my life played a vital role into my ability to finishing the thesis project. As far as being acclimated into the lab, I would like to express gratitude towards my lab manager, Thirunavukkarasu Annamalai, for helping me become familiar with good laboratory practices and instructions of the experimental protocols related to the project. My current/former lab members also contributed to the project and helped me out with basic lab duties such as proper lab etiquette.

The list goes with individuals who have supported me. I would like to thank my committee members, Dr. Yuan Liu, and Dr. Jaroslava Miksovska, for feedback on my project and emphasis on developing good work discipline as I pursue post-graduate opportunities. In addition, the collaborators who played a vital role in keeping this project afloat will not go unnoticed for their hard-working efforts. Although they are not involved in the lab, no words cannot describe the amount of support given by my family. They made sure that I was never short of a meal and lent a hand, whether it was for emotional support or for relieving some of my finances. Dr. Carmen Ballestas, my primary care oncologist, and the Pediatric Oncology Department at Joe Dimaggio Children's Hospital were the main driving force in helping me overcome by 5-year battle with leukemia. Their passion and devotion to developing the best quality care serves as a constant reminder that what they do daily has more meaning than just a career to make a salary out of.

Most importantly, I would not be able to overcome the obstacles with graduate school and my project without the input of my research advisor, Dr. Yuk-Ching Tse-Dinh. Not only did she offer her guidance and decades of her expertise into the development of my project, but she helped me accommodate enough time in my hectic work schedule to meet the demands of the program. I will not forget this opportunity to meet an inspirational figure who not only has an appreciation for her research but wishes for nothing but the best for her students.

ABSTRACT OF THE THESIS

IDENTIFICATION OF HUMAN TOPOISOMERASE I POISON AND CATALYTIC INHIBITORS

by

Christian Madeira

Florida International University, 2021

Miami, Florida

Professor Yuk-Ching Tse-Dinh, Major Professor

Inhibition of human topoisomerase I has been shown to reduce excessive transcription of PAMP-induced genes based on prior studies, which offers a solution to offset complications of sepsis such as tissue damage and organ failure. The enzyme resolves the topological constraints on DNA that encodes these genes. The aim of this study is to identify human topoisomerase I (HTop1) inhibitors that can a.) prevent relaxation of negative supercoiled plasmid DNA by HTop1 and b.) reduce HTop1 binding to DNA and formation of covalent cleavage complex (HTop1cc) utilizing a novel yeast screening system. Top hits from *in-silico* screening conducted by our collaborator showed moderate to no inhibition. The natural products anthocyanidins, delphinidin and cyanidin chloride, were shown to partially restore growth of yeast expressing a lethal HTOP1 mutant by preventing HTop1cc. Myricetin inhibited yeast growth in the presence and absence of HTop1 overexpression, which requires further investigation. This novel yeast screening approach can offer potential insights to the inhibitor's mechanism of action.

TABLE OF CONTENTS

LIST OF TABLES

TABLE OF FIGURES

FIGURE PAGE

CHAPTER 3

ABBREVIATIONS AND ACRONYMS

ATP	adenosine triphosphate
CPT	camptothecin
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dsDNA	double-stranded DNA
E Coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
HTop1	human topoisomerase 1 protein
HTOP1	human topoisomerase 1 gene
HTOP1cc	human topoisomerase 1 gene
IC_{50}	inhibitory concentration at half maximum value
KLD	kinase, ligase and DpnI
Lk	linking number
NEB	New England Biolabs
OD600	optical density measurement at 600 nm
PAMP	pathogen associated molecular patterns
PCR	polymerase chain reaction
SC-U	synthetic complete without uracil
SDS	sodium dodecyl sulfate
ssDNA	single-stranded DNA
TDP1	tyrosine phosphodiesterase I
Top1cc	topoisomerase I cleavage complex

Top1	topoisomerase I protein
TPT	Topotecan
WT	wild type
TRIS	2-Amino-2-(hydroxymethyl)-1,3-propanediol
YTOP1	*Saccharomyces cerevisiae* topoisomerase 1 gene
YTop1	*Saccharomyces cerevisiae* topoisomerase 1 protein

CHAPTER I: INTRODUCTION

A. STRUCTURE AND MECHANISM OF HUMAN TOPOISOMERASE I

Human topoisomerases resolve topological constraints during DNA transcription and replication. There are two classes, type I and II, that cleave a single or double strand of DNA, respectively. Subfamilies such as IA, IB, and IIA type topoisomerases share similar structural and mechanistic features to their class (Pommier, 2013). However, all topoisomerases share a common property: relax supercoiled DNA by the formation of a phosphotyrosine intermediate (Figure 1.1) with the 5' or 3' end of the DNA segment following cleavage of the DNA (Wang, 2002). Formation of the intermediate is followed by strand passage of the intact strand through the cleaved strand before religation. In the case of human topoisomerase I (HTop1), a type IB topoisomerase, the enzyme forms a 3' phosphotyrosine covalent intermediate with cleaved DNA (Cretaio et al., 2007, Krough and Shuman, 2000).

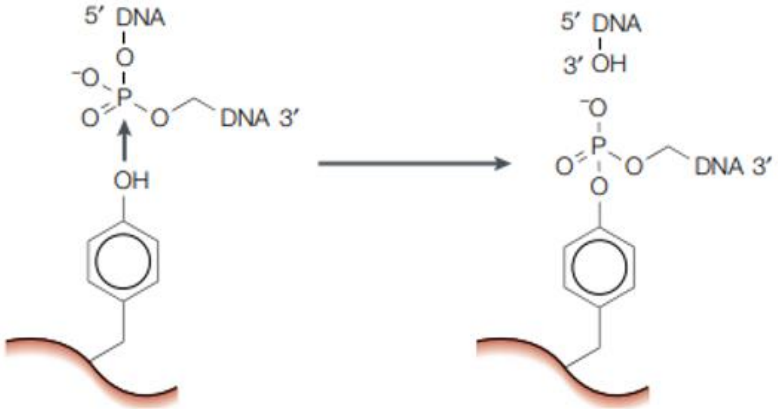


Figure 1.1 Transesterification between active site tyrosine and DNA phosphate group to create Top1cc. Reaction shown is catalyzed by type IA or IIA topoisomerases. In the case with type IB, the leaving group is a 5' OH and a 3' phosphoryl group is linked to the active site tyrosyl group (Wang, 2002).

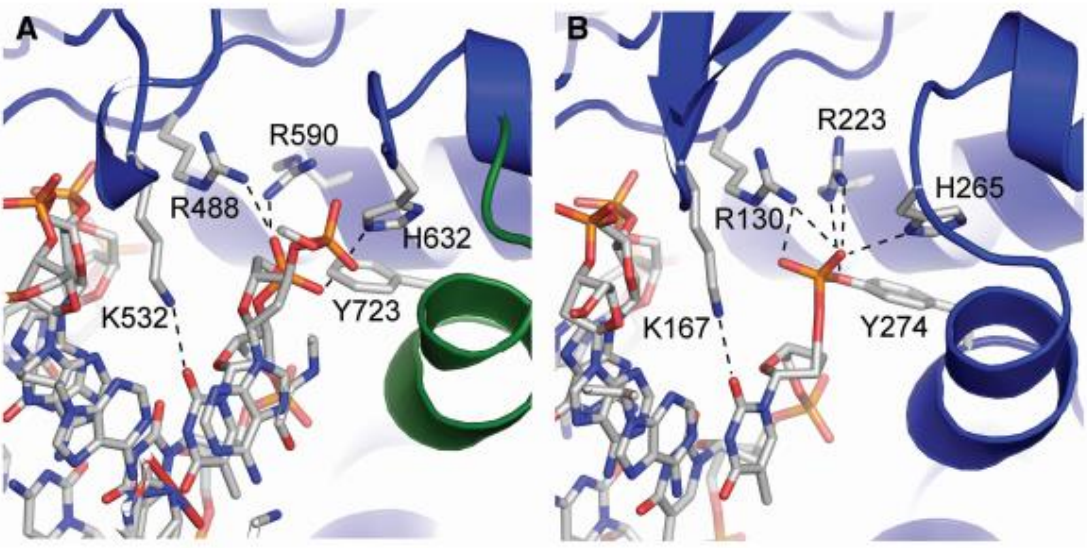


Figure 1.2 Similarities of type 1B topoisomerases HTop1T723Y mutant (Redinho et al. 1998) in a non-covalent complex is shown to the left. Variola virus Top1 (Corless et al 2017) is shown to the right. Each diagram highlights the surrounding active site residues.

Their mechanism of relaxaton consists of controlled rotation of the broken 5' end around the intact 3' strand of DNA (Pommier, 2013). Human topoisomerase I has several elements: core subdomains I-III and a C-terminal domain that binds DNA (Figure 1.3). The structure of HTop1 interacting with DNA is analogous to the structure of polymerase interacting with DNA. Subdomains I and III act as a hand, while subdomain III and the C-terminal domain act as a palm to position the DNA for cleavage. The grip loosens to allow the intact DNA to rotate around the cleaved strand (Berger, 1998). The amino acid sequence of human topoisomerase I is conserved in multiple eukaryotic species expressing topoisomerase I. Residues around the active site region are identical among Top1B from all species (figure 1.2). (Gupta et al., 1995, Baker et al., 2009).

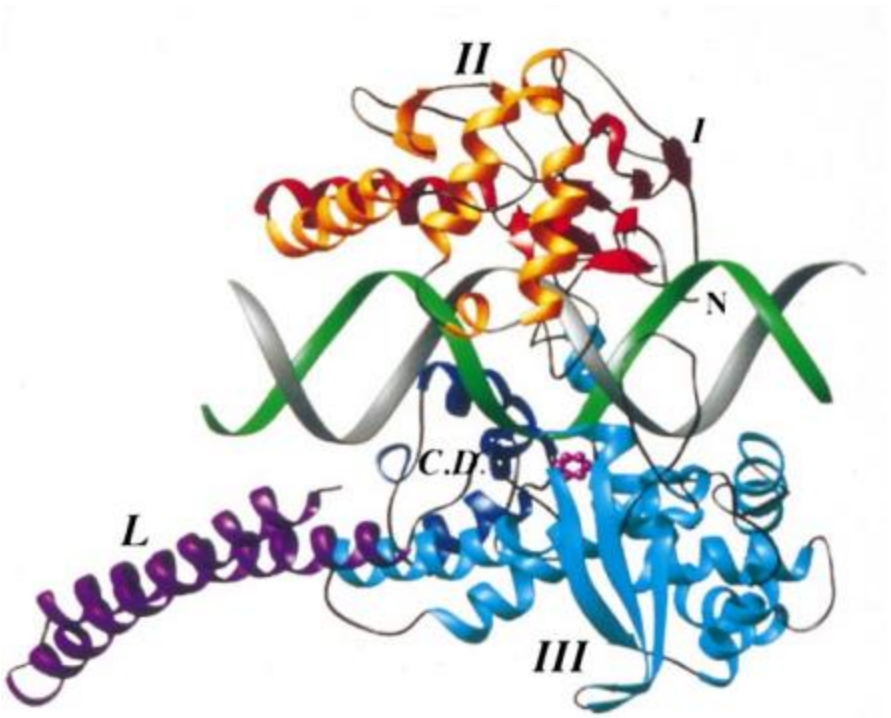


Figure 1.3. RIBBONS generated structure of 70 kDA human topoisomerase I bound to DNA. The following is noted: I-III: Core subdomains I (red), II (yellow), and III (light blue). Linker domain (purple), c-terminal domain (dark blue) is also noted. Active site tyrosine is substituted for phenylalanine, indicated by magenta spheres. (Berger, 1998)

B. TOPOISOMERASES AND DNA SUPERCOILING

DNA supercoiling occurs when DNA becomes overwound or underwound in response to DNA binding proteins. The proteins are involved in bending, transcribing, and replicating DNA (Coreless et al., 2017). The torsional stress from supercoiling can create axial stress on DNA molecules, causing strands to writhe or cross each other.

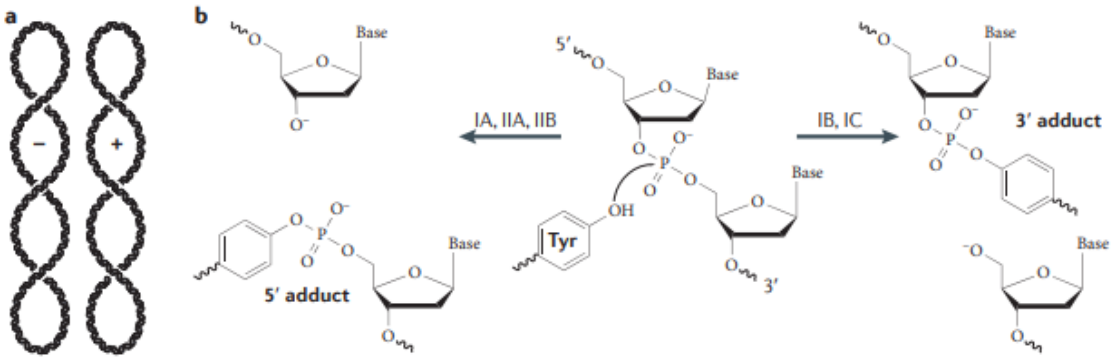


Figure 1.4 DNA Topoisomerases resolve DNA supercoiling. a. Different forms of DNA supercoiling. Negative and positive supercoiling are indicated by wrapping in the clockwise (right-handed) or counterclockwise (left-handed) direction, respectively. As shown in b., Type IA, IIA and IIB topoisomerases form an intermediate product with the 5' end DNA following nucleophilic attack of the active site tyrosine residue. On the other hand, type IB and IC forms a product with the 3' end of the DNA (Vos et al., 2011).

Type I topoisomerases change the linking number of supercoiled DNA by units of 1 and remove supercoiling (Figure 1.4). The linking number (Lk) is defined as the number of times a DNA strand winds in a right-handed direction around the DNA helix on a fixed plane. Unlike type II topoisomerases, type I topoisomerases do not resolve interlinked ssDNA since their catalytic activity involves ssDNA cleavage. An interesting feature of topoisomerases is that they conserve the bond energy of DNA molecule since only one bond of the sugar-phosphate DNA backbone is nicked (Deweese et al. 2009).

C. APPLICATIONS OF HUMAN TOPOISOMERASE I INHIBITORS

Clinical settings utilize anti-cancer and anti-bacterial agents that targets topoisomerases. Poison inhibitors such as Camptothecin (CPT) act by trapping the intermediate cleavage complex of formed between the DNA and enzyme (Figure 1.5).

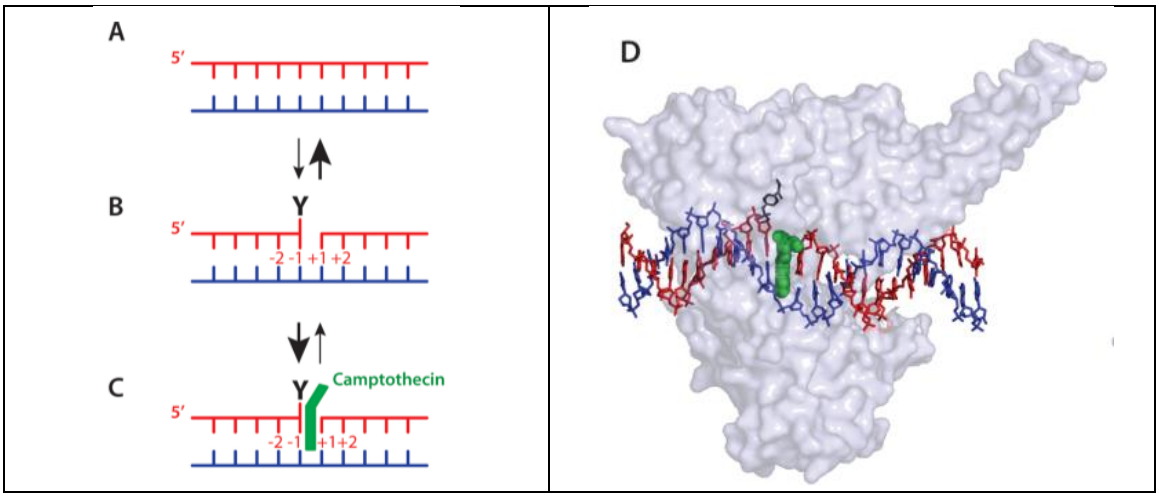


Figure 1.5. Mechanism of CPT on human topoisomerase I. Cleavage (A to B) and religation (B to A) of DNA by HTop1 followed by reversible binding of CPT (B to C). 3D visualization of ternary complex formed between CPT. DNA and HTop1 (d) is shown to the right (Pommier, 2013).

Rialdi et al. explored the potential use of topoisomerases as a target for the treatment of hyper immune response of host cells that occurs during sepsis. Sepsis begins from the onset of inflammatory response to pathogen associated molecular patterns (PAMPs). Pathogen recognition receptors found in the host immune cells generate an immunological response cascade when encountering PAMPs. This leads to local and systematic tissue injury caused by to uncontrolled PAMP-induced gene expression. Camptothecin and Topotecan, topoisomerase I poison inhibitors, have been shown to reduce the host response against pathogens by reducing expression of PAMP-induced genes without changing the house keeping gene expression nor inflicting damage to the cell (Rialdi et al. 2016). These researchers also observed that CPT treatment significantly increased the survival of mice infected with influenza and *Staphylococcus aureus.* Top1 knockout studies in A549 cells infected with PRSΔN81 showed decreased expression of

84 genes in the infected cells relative to control. Decreased expression of the genes validates Top1 as a potential target for suppressing the cytokine storm. The results from the study present an encouraging cause for exploring the possibility of using human Top1 inhibitors as a treatment option for sepsis and SARS-Cov-2 (Ho et al, 2020). However, all known approved Top1 inhibitors in clinical settings are cytotoxic anticancer drugs that are not suited for sepsis treatment in humans (Esselen et al., 2011). They are derived from Camptothecin (CPT), a quinolone alkaloid extracted from the bark of *Camptotheca acuminata,* which inhibits the enzymatic activity of Top1 by stabilizing the Top1 cleavage complex (Top1cc) and prevents religation. Inhibition that involves the stabilization of the Top1cc is known as poison inhibition. One consequence of poison inhibition is cell death since the trapped Top1cc collide with moving replication forks to create dsDNA breaks (Chrencik et al., 2004). These CPT derived compounds are poison inhibitors unlike catalytic inhibitors, which may inhibit DNA binding and cleavage, without causing cytotoxic increase of DNA breaks (Pommier et al., 2013). These types of inhibitors prevent HTop1cc formation by acting upstream of the DNA cleavage reaction (Yu et al., 2019). Dose limiting toxicities of CPT-derived compounds and related cytotoxic poison inhibitors are concerns for therapeutic treatment in sepsis. Therefore, there is a need for novel clinical drugs that will target topoisomerase I in humans as catalytic inhibitors.

D. NATURAL PRODUCTS AS TOPOISOMERASE INHIBITORS

Flavonoids are phenolic structures derived from plants. Their general structure (Table 1.1) consists of two benzene rings bonded to a pyran ring. Different classes of structures have unique substitution patterns of the C rings. and differ by substitutions on the A and B ring (Kumar and Pandey, 2013). Hydroxyl substitution, especially at C-3, C-7, C-3' and C-4' position in flavonoids were shown to inhibit type I and II topoisomerases and may have clinical applications in cancer chemotherapy (Constantinou et al., 1995).

Anthocyanidins, a group of aglycone anthocyanins (Figure 1.6), make up colored pigments in food, and in pharmaceutical ingredients with potential health benefits. Aside from their anti-oxidative and anti-inflammatory properties, they affect cell cycling and differentiation (Webb et al., 2008).

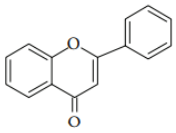
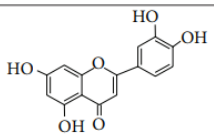
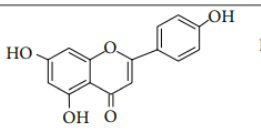
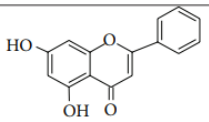
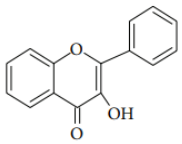
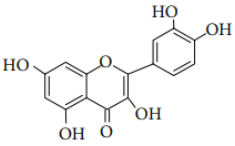
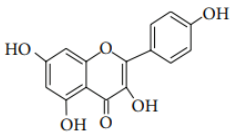
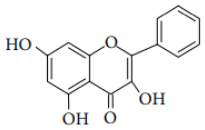
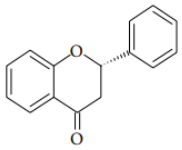
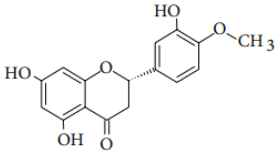
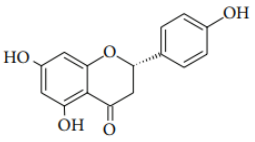
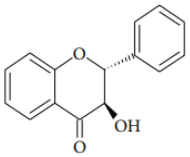
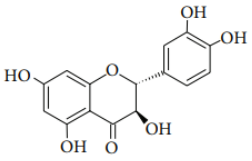
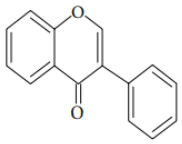
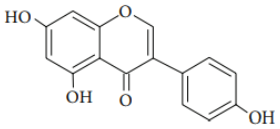
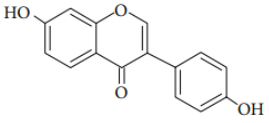
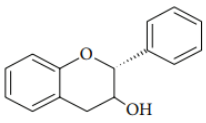
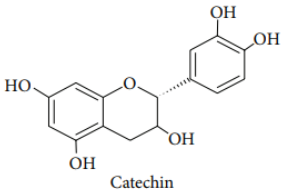
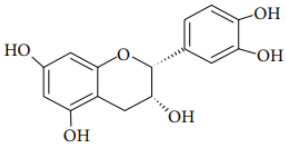
Group of flavanoid	Structure backbone	Examples		
Flavones		 Luteolin	 Apigenin	 Chrysin
Flavonols		 Quercetin	 Kaempferol	 Galangin
Flavanones		 Hesperetin	 Naringenin	
Flavanonol		 Taxifolin		
Isoflavones		 Genistein	 Daidzein	
Flavan-3-ols		 Catechin	 Epicatechin	

Table 1.1. Different classes of flavonoids and their derivatives (Kumar and Pandey, 2013)

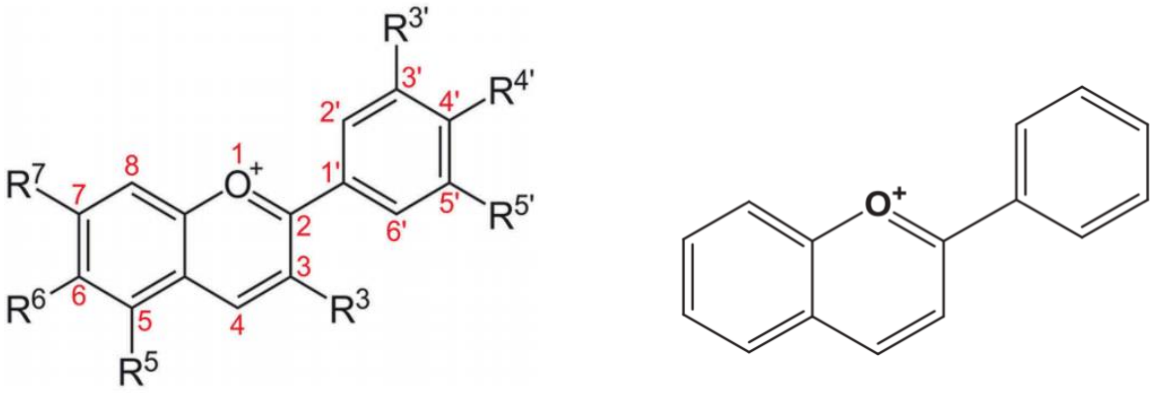


Figure 1.6. Basic anthocyanin and flavylium ion structure (Khoo et al., 2017)

Despite the numerous health benefits posed from anthocyanidins or flavonoid compounds, overconsumption of these compounds has posed health risks. It is known that certain flavonoids can inhibit topoisomerase I and II enzymes or modulate interactions with topoisomerase II poisons. However, several issues exist with identifying topoisomerase I inhibitors: uncertainty with distinguishing between catalytic inhibition, poisoning, and DNA intercalation, in addition to the stability of flavonoids in aqueous solutions (Esselen et al., 2009, Webb et al., 2008).

E. IN SILICO SCREENING OF SMALL MOLECULE INHIBITORS OF HTOP1

Virtual screening and molecular dynamics, computational approaches used in structure-based drug discovery, allows one to predict the binding affinity of ligands to receptor molecules. Inhibition of HTop1 has been a goal in the development of cancer chemotherapeutics. Ligand-enzyme complementarity, proximity of molecule to active site or nearby residues serves as criteria for the selection process. Challenges with discovering HTop1 inhibitors include structural dynamics and inability to validate cleavage complex formation of HTop1 and DNA (Xin et al., 2017; Tiwari et al., 2019).

F. POTENTIAL DRUG REPURPOSING TO IDENTIFTY HTOP1 CATALYTIC INHIBITORS

To develop new therapeutic practices, inhibitor candidates that interact with polymerases were obtained from Boehringer Ingelheim to explore the possibility of drug repurposing. Their negative controls, which report significantly lower activity, are also explored. If selected compounds show inhibition of HTop1, analogs can also be explored for inhibition in the future.

Compound	Function
BI-207127	Inhibitor of HCV polymerases (Chen et al., 2015)
BI-7656	Negative control of BI-207127
BI-2540	Inhibitor of NNRTs (Sarafianos et al., 2009)
BI-2439	Negative control of BI-2540

Table 1.2. Boehringer Ingelheim compounds tested for drug repurposing.

CHAPTER II: INHIBITION OF NEGATIVELY SUPERCOILED DNA RELAXATION BY PURIFIED HTOP1

A. INTRODUCTION

The catalytic activity of HTop1 can be assayed by its ability to relax supercoiled DNA. The enzyme is ATP-independent and does not require a divalent ion, such as Mg^{2+} for activity. These properties will be used to distinguish the activity of HTop1 from other human topoisomerases that all require ATP and/or divalent ions (Corbett and Berger, 2004; Schoeffler and Berger, 2008). Human topoisomerase I relaxes both positive and negative supercoiling. Negative supercoiling allows strand separation because the underwound DNA allows its replication and expression. Compared to positive supercoiling Protein elements that bind to the replication origin and gene promoter on DNA have better access when DNA is negatively supercoiled., increasing the rate of DNA replication and transcription. Positive supercoiling inhibits DNA replication and transcription since the overwound DNA blocks access to binding elements (Deweese et al., 2009, Vos et al.,2011). The potential inhibitors will be verified for relaxation inhibition using purified HTop1 in agarose gel electrophoresis-based assay using the standard procedures used previously in our lab (Sandhaus et al. 2016).

B. MATERIALS AND METHODS

The following compounds obtained from the *in silico* screening, NSC372499, NSC158549, and NSC379651 have been obtained from the NCI Developmental Therapeutics Program. Fisetin (catalog no.: A10388-500), myricetin (catalog no.: A10615-50), galangin (catalog no.: A14607-10), daidzein (catalog no.: A10282-500),

biochanin A (catalog no.: A10146-200), diosmetin (catalog no.: A10326-25), luteolin (catalog no.: A10541-10), pinocembrin (catalog no.: A14759-10), naringenin (catalog no.: A10625-25), and cyanidin chloride (catalog no.: A10625-25) were purchased from AdooQ Bioscience. BI-2540, BI-2439, BI-207127, and BI-7656 were obtained from Boehringer Ingelheim under the opnMe program. 10-20 mM stocks of the compounds were made in ~99% DMSO and stored under -30^{o}C.

Purified HTop1 was either purchased from TopoGen or purified from yeast expressing recombinant HTop1 in our lab. 0.5 U of HTop1 was mixed with 10X reaction buffer by TopoGen containing 2 M Tris-HCL at pH 8.0, 0.5 M EDTA, 5M NaCl, 100 mM spermidine, 100 mg/mL BSA and 100% glycerol. The compounds were added to the enzyme suspended in the buffer, followed by 200 ng of negative supercoiled DNA, for a final volume of 20 µL. The reactions were terminated with 6x SDS stop buffer (6% SDS, 6 mg/mL bromophenol blue, 40% glycerol) and was analyzed via 1% agarose gel electrophoresis at 20-25 V. The bands were imaged using the ProteinSimple AlphaImager Mini following staining in a 1 µg/mL solution of EtBr for 30-45 minutes and rinsing for 10-15 minutes in deionized water.

C. RESULTS AND DISCUSSIONS

Detection of relaxation inhibition of HTop1

The test compounds were assayed for relaxation inhibition of HTop1 between the concentrations of 15.625 µM – 250 µM. The concentration needed to inhibit 50% of HTop1 relaxation activity (IC_{50}) was determined for the compounds that revealed activity at concentrations in the test range of 15.625 µM – 250 µM.

The intensity/analytical measurement tools from *Image J* and/or *AlphaView SA* were used to calculate the values. The percent of supercoiled DNA was expressed as the ratio between the intensity of supercoiled DNA band in control reaction with no enzyme versus the intensity of supercoil DNA in a reaction containing HTop1 and DNA with a certain concentration of compound. Each intensity value is calculated as the band intensity with background intensity subtracted. Dimethyl sulfoxide (DMSO) has been shown to affect the relaxation of HTop1 (Lv et al., 2015). Therefore, the band containing the enzyme, DNA and DMSO will be used as a negative control for no inhibition. Compounds not showing activity within this concentration range were not tested further.

Testing of *in silico* screening hit compounds

In silico screening hit compounds identified from a set of compounds in the NCI collection as potential HTop1 inhibitors by our collaborator Dr. P. Tiwari at Georgetown University were tested for inhibition of HTop1 relaxation activity. Tiwari has previously identified bacterial topoisomerase I inhibitors using a similar *in silico* screening approach (Tiwari et al., 2020). The results showed no relaxation inhibition of HTop1 between the concentrations of 15.625-250 µM when compared to the DMSO negative control (Figure 2.1.). No further trials for the *in silico* screening hot compounds were performed but they were fully evaluated for growth inhibition (results described in Chapter 3) to see if the presence of the inhibiters affected HTop1's ability to bind to DNA. Only DNA present in lane 1 (Figure 2.1a.) and the reaction in lane 2 with CPT interacting with HTop1 and DNA shows inhibition of HTop1 relaxation indicated by the presence of supercoiled DNA.

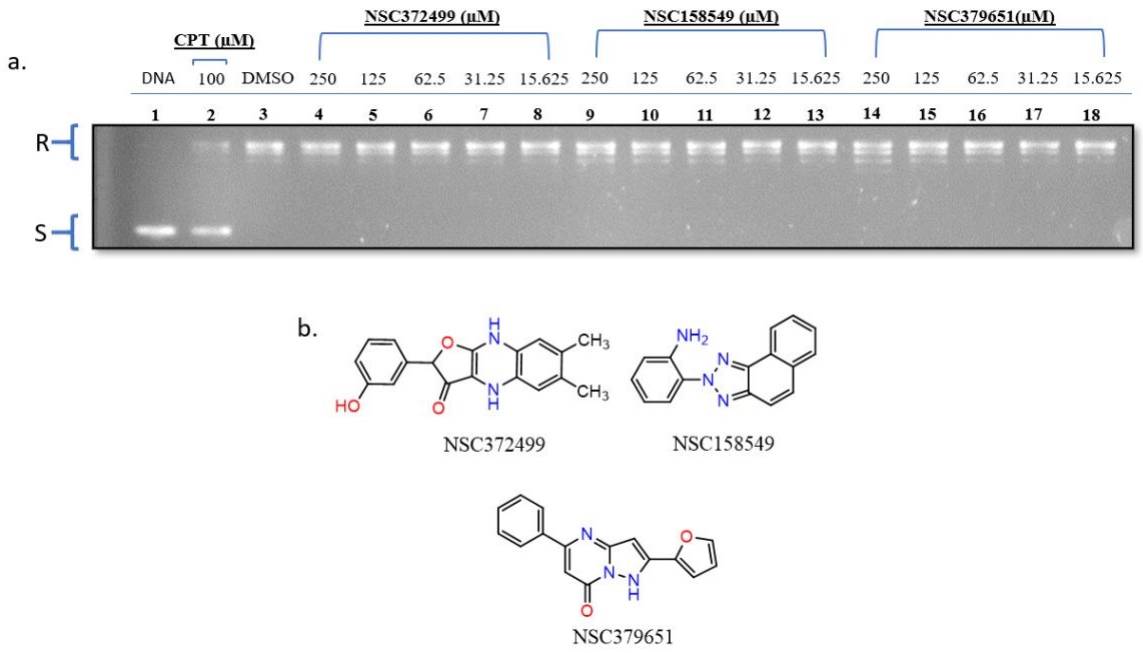


Figure 2.1. *In Silico* Screening hit compounds do not inhibit relaxation of HTop1. a.) NSC372499, NSC158549 and NSC379653 are tested within the concentrations of 0-250 µM. R: Relaxed DNA, S: supercoiled DNA. b.) Structure of compounds

Inhibition of HTop1 Relaxation Activity by Natural Products

The natural products delphinidin and cyanidin chloride exhibited IC_{50} values between the concentrations of 125-500 µM (figure 2.2.) These compounds differ from each other by the presence of a hydroxyl group in the 3' position of the pyran ring in the case of cyanidin chloride, and in the 3' position of the B ring in the case of delphinidin chloride (Habermeyer et al., 2005). The differences in the hydroxyl group substitutions can be a contributing factor to their differences in relaxation inhibition of HTop1 seen in the results.

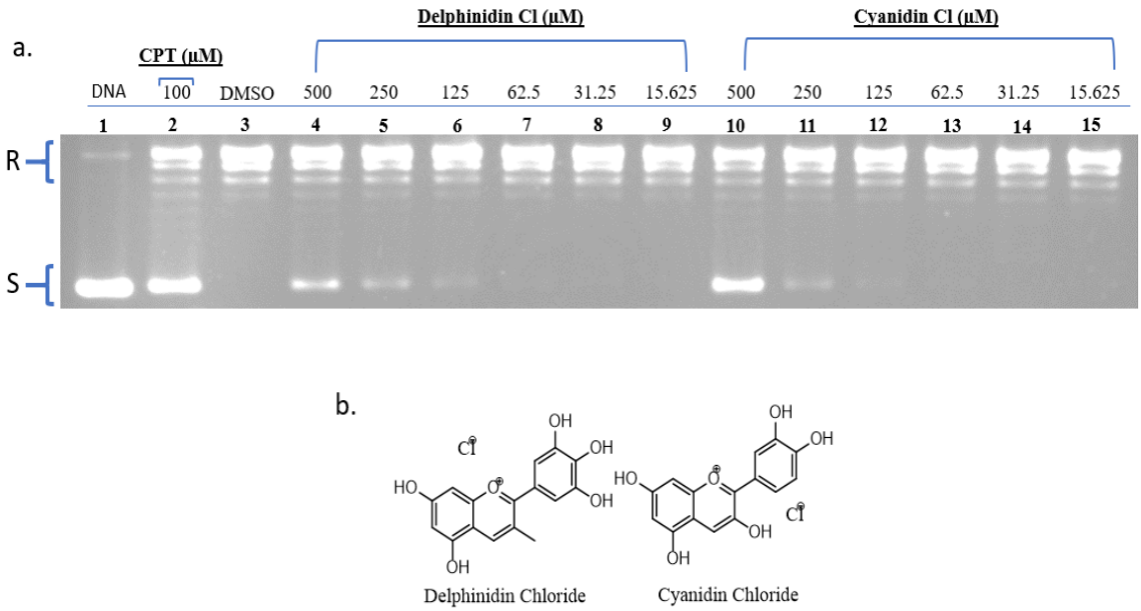


Figure 2.2. Relaxation inhibition of HTop1 in the presence of anthocyanidins a.) Delphinidin and cyanidin chloride are tested within the concentrations of 0-500 µM. b.) Structure of compounds R: Relaxed DNA, S: supercoiled DNA.

Out of the flavonoids tested, only myricetin showed complete inhibition with an IC_{50} between 31.25-62.5 µM. The other flavonols, as well as flavones and isoflavones tested, exhibited no inhibition. Topotecan (TPT) is an analog of CPT (Figure 2.3.b) that acts also as a HTop1 poison inhibitor. This was used as a positive control here (Mabb et al., 2016, Koster et al., 2007). Several studies reported type I and II topoisomerase inhibition from Myrcetin, mainly caused hydroxyl group substitutions at C-5, C-3', C-4' and C-5' (Constantinou et al., 1995, Bendele and Osheroff, 2007). In addition, it is shown to induce high levels enzyme-DNA complex for both enzymes (Lopez-Lazaro et al., 2009) It is possible that the hydroxyl oxygen atoms on the A and B ring could interact with nearby active site residues of HTop1, as evidenced in molecular docking studies (Xin et al., 2017).

Relaxation inhibition of supercoiled DNA by topoisomerase I and II have been reported for the flavonoids. However, the reaction conditions differ considerably and as a result, the results of the study may not agree with prior studies. For instance, Webb et al. reported Top1 poisoning with the following flavonoids at 100 μM (from greatest to least): myricetin, luteolin, fisetin, daidzein, diosmetin and naringenin. These researchers also monitored the change in absorbance values of the compounds, with different pH values, from UV-visible spectrophotometry studies. They have discovered that the compounds' solubility and stability in aqueous solutions is affected by a change in pH value.

Chowdhury et al. reports catalytic inhibition of Top1 with luteolin and an increase in catalytic activity when the drug is incubated with the enzyme. On the other hand,preincubation with the drug and DNA before the addition of the enzyme was shown to reduce the inhibitory effect. Single hydroxyl group substitutions were also investigated with luteolin. Quercetin differs from luteolin by a hydroxyl group present at C-3 and can prevent religation of Top1 (Corless et al., 2017). Similar to the relationship between luteolin and quercetin,

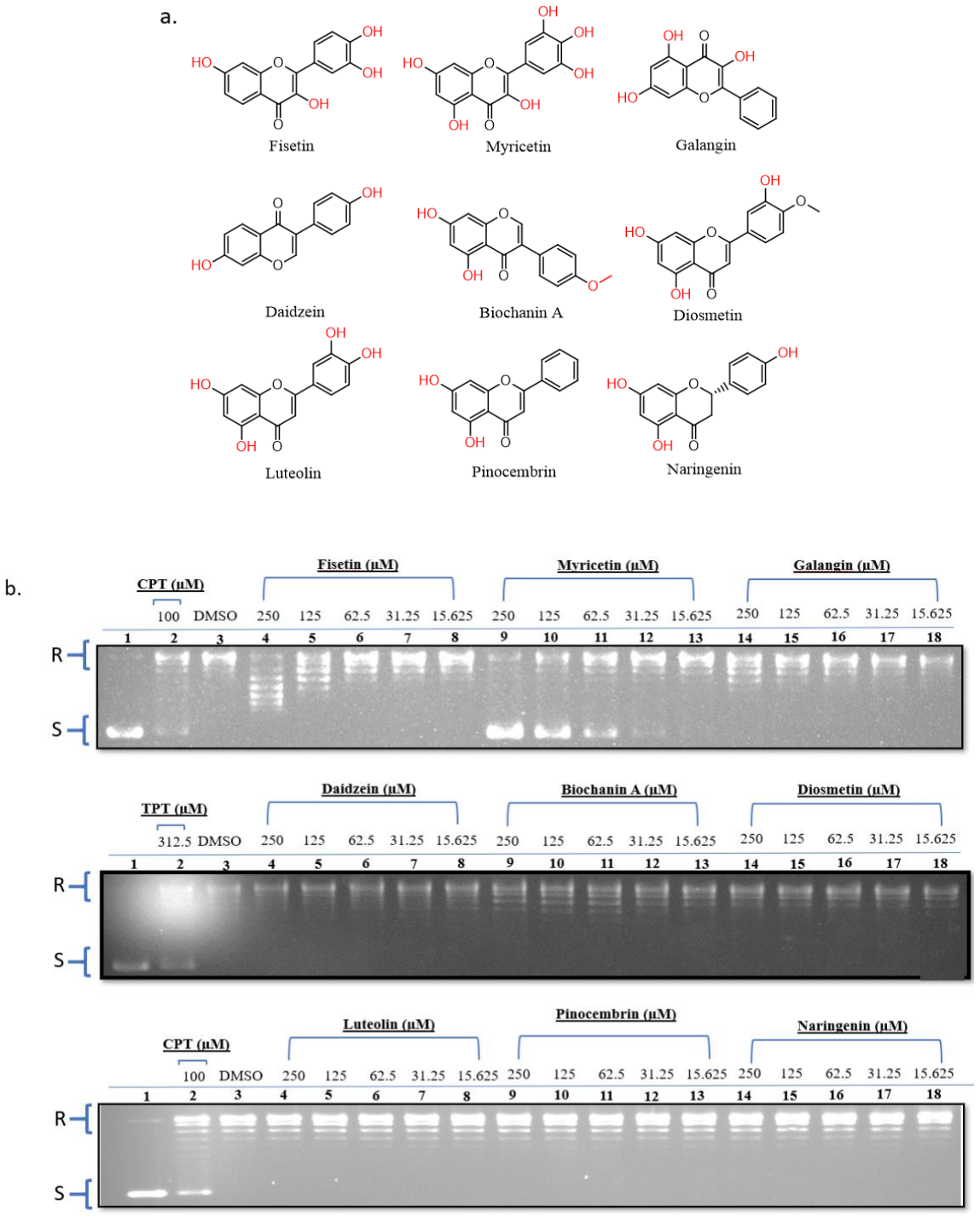


Figure 2.3 Relaxation inhibition of HTop1 in the presence of flavonoid compounds. a.) Structure of compounds. b.) Compounds are tested within the concentrations of 0-250 μM. R: Relaxed DNA, S: supercoiled DNA.

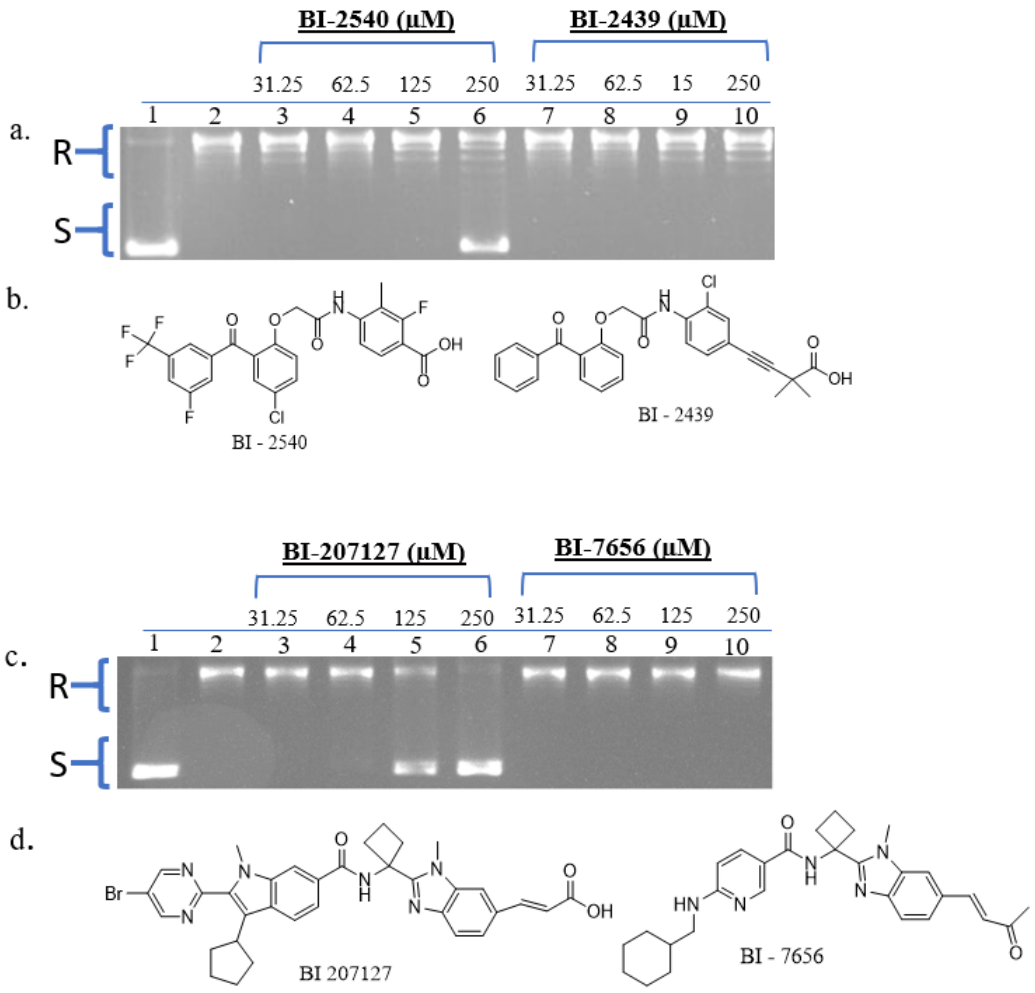


Figure 2.4. Relaxation inhibition of HTop1 in the presence of BI compounds and their negative controls Compounds are tested within the concentrations of 0-250 µM (a and c) R: Relaxed DNA, S: supercoiled DNA. Structure of compounds (b and d).

Boehringer Ingelheim makes several their approved drugs and drug candidates free of charge to academic researchers under the OpnMe program to encourage innovative use of these molecules. We obtained two compounds with inhibition of polymerases as mode of action for testing against HTop1 because of the similarities in interaction with DNA as described in Chapter 1. Compounds BI-2540 and BI-207127 shows inhibition starting at 250 µM and 125 µM, respectively (figure 2.4. a-b).

On the other hand, their negative controls display no inhibition (figure 2.4. c-d). The potency of HTop1 inhibition is much weaker than the inhibition against the polymerase targets (IC50 in nM range).

To further characterize the inhibitor candidates tested, DNA cleavage assay of HTop1 can be utilized. Poison inhibitors such as CPT stabilize the HTop1cc. In the cleavage assay, the level of these complexes can be quantified by the amount of broken DNA formed in the presence of the test compound and the enzyme. Separation of DNA strands at the ssDNA break reveals open, circular DNA that can be separated from relaxed DNA and linear DNA by gel electrophoresis (Nitiss et al., 2012). Previous experiments observed that cyanidin and delphinidin chloride diminished the stabilization of Top1cc in the presence of poison inhibitor CPT (Habermeyer et al., 2005) suggesting that they may be catalytic inhibitors instead of poison inhibitors. The yeast growth inhibition assay in Chapter 3 will be used verify this in further testing of these inhibitor candidates to distinguish between poison inhibitors that stabilize the Top1cc and catalytic inhibitors that prevent the formation of Top1cc.

CHAPTER III: ASSAY OF GROWTH INHIBITION OF SACCHAROMYCES CEREVISIAE EKY3 STRAIN EXPRESSING WILD-TYPE OR T718A MUTANT RECOMBINANT HUMAN TOPOISOMERASE I

A. INTRODUCTION

Development and optimization of yeast screening assay

Saacharomyces cerevisiae contains a type IB topoisomerase I protein (YTop1) similar to HTOP1 but not essential for its viability (Goto and Wang, 1985). Yeast cells with YTOP1 substituted with recombinant human HTOP1 develops sensitivity to CPT drug treatment (Bjornsti et al. 1989). Other poison inhibitors of HTop1 are expected to result in lethality when wild-type recombinant HTop1 is overexpressed in the yeast strain lacking YTop1. Mutant HTop1 with threonine 718 near the active site substituted for alanine (HTop1T718A) causes lethality to yeast when overexpressed (Fiorani et al. 1999) since this mutation mimics the action of a HTop1 poison inhibitor to stabilize the Top1 cleavage complex (Top1cc), resulting in accumulation of breaks on chromosomal DNA. This HTop1T718A mutant induced lethality was used in this study to screen for novel Top1 inhibitors. A catalytic inhibitor of TOP1 DNA binding or cleavage activity will prevent lethal Top1cc formation by the HTop1718A mutant which should in turn rescue the yeast cells from growth inhibition. The HTop1 catalytic inhibitor can either bind competitively to the same site as DNA or at a separate site to act as an allosteric regulator (Soren et al., 2020), changing the conformation of HTop1 so that the enzyme cannot bind or cleave DNA.

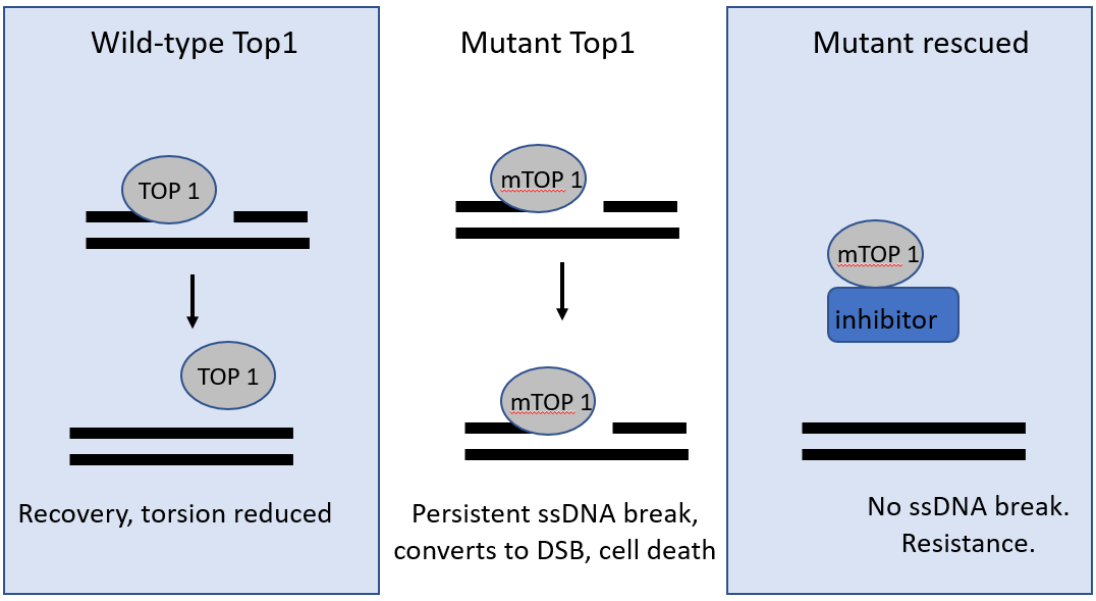


Figure 3.1. Rescue of lethality from mutant Top1cc accumulation under the presence of catalytic inhibitor

Potential Top1 inhibitors have been identified via *in silico* screening conducted by our lab's collaborator Dr. Purushottam Tiwari at Georgetown University using procedures like those employed for identification of *Escherichia coli* topoisomerase I (Tiwari et al., 2019). These small molecules have been obtained from the NCI Developmental Therapeutics Program and are being tested in the growth inhibition assay based on recombinant wild-type or mutant recombinant HTop1 overexpressed in yeast here, in addition to the assay for inhibition of the relaxation activity of HTopI in the biochemical assay as described in Chapter 2.

B. MATERIALS AND METHODS

Preparation of plasmid constructs

pYES2.1 expressing wild type (WT) human topoisomerase I under the control of the galactose inducible GAL promoter (suppression by dextrose) was custom synthesized by Genscript. HTOP1T718A mutant was created with the Q5 site-directed mutagenesis kit (NEB) and the template plasmid for site directed polymerase chain reaction (PCR) is PYES2.1-HTOP1, which expresses the wild type HTOP1. The forward and reverse primers 5'-TCGCCTGGGAGCCTCCAAACTCA-3' and 5'-ATCTGTTTATTTTCCTCTCGGTCTGTG-3', respectively, was used for the site-directed PCR reaction. To prepare template DNA for site-directed mutagenesis, 2 µL pYES2.1-HTOP1 plasmid DNA was added to 25 µL of NEB Turbo competent cells (from New England BioLabs), followed by incubation on ice for 30 minutes and heat shock at 42^{o}C for 30 seconds. 950 µL of the LBN media (Luria Broth with 5g/L NaCl) was added to the mixture of plasmid and cells. Following incubation at 37^{o}C with shaking at 200 rpm, 100 µL of the culture was spread on LBN/Carbenicillin (100 mg/mL) plates and incubated overnight at 37^{o}C to isolate the colonies of transformed cells. Plasmid purification of pYES2.1-HTOP1 plasmid DNA from the transformed *E. coli* was conducted using the NEB Monarch® Plasmid Mini Prep Kit. The mutagenesis primers were resuspended to 100 µM using TE buffer (10mM Tris HCl, pH 8, 1mM EDTA), 1:10 diluted with H_2O and used in 25 µL NEB Q5 polymerase site-directed mutagenesis PCR reaction (5 ng/mL template DNA). Cycling conditions are listed in table 3.1.

STEP	TEMPERATURE (ºC)	TIME
Denaturation	98	10 seconds
25 Cycles	98 68 72	10 seconds 30 seconds 4 minutes, 15 seconds
Final Extension	72	5 minutes
Final Hold	4	-

Table 3.1. PCR cycling conditions for site directed mutagenesis of pYES2.1-HTOP1

The PCR product was further treated with Kinase, ligase, and DpnI (KLD) enzyme mix as described by manufacturer (New England Biolabs). DpnI was used to digest the template DNA. The reactions were terminated by 5x stop buffer (50% glycerol, 50 mM EDTA at pH 8.0, and 0.5% v/v bromophenol blue). The final PCR product was verified by electrophoresis in 1% agarose gel followed by ethidium bromide staining. After successful amplification, the pYES2,1-HTOP1 DNA expressing recombinant HTOP1 with T718A mutation was then extracted from agarose gel (Zymoclean® Gel DNA Recovery Kit) and transformed into competent NEB turbo *E. coli* cells. Volumes of 40-100 µL of transformation mixtures were plated on LBN/Carbenicillin (100 mg/mL) agar plates and incubated overnight at 37ºC.

The resulting single colonies from the plates were cultured in 5 mL of LBN/Carbenicillin (100 µg/mL) overnight for plasmid prep. The resulting plasmid pYES2.1-HTOP1T718A was extracted using the NEB Monarch® Plasmid Mini Prep Kit and commercially sequenced through Eurofins Geonomics to confirm the presence of the T718A mutation, and no other mutations in the HTOP1 coding sequence.

Growth Inhibition of EKY3-pYES2.1HTop1 and EKY3-pYES2.1HTop1T718A

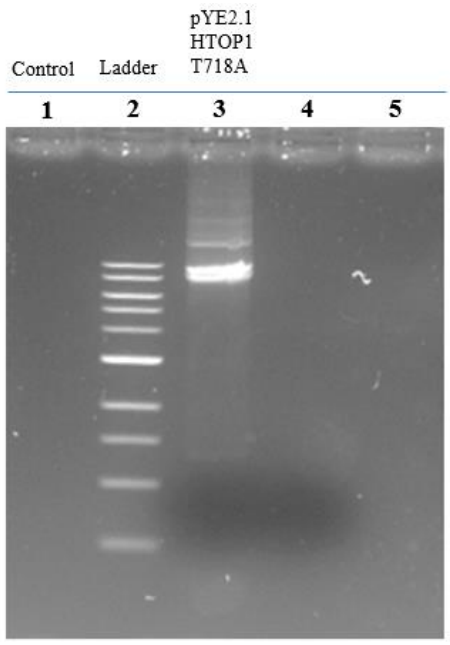
Sequenced verified construct was transformed into competent EKY3 cells by our collaborator Dr. Aaron Welch. Overnight cultures of EKY3-pYES2.1HTop1, EKY3-pYES2.1HTop1T718A in synthetic complete media minus uracil (SC-U) + 2% dextrose was grown in 30^{o} C shaker for 18 hrs. These cultures were diluted at 1:50 into 3 mL of SC-U + 2% raffinose and grown overnight under the same conditions. The cultures were 1:10 diluted in both types of cultures: 1.8 mL of SC-U + 1% galactose + 0.25% raffinose to induce recombinant HTop1 expression, or SC-U + 1% dextrose + 0.25% raffinose to suppress recombinant HTop1 expression. OD600 for each was adjusted to 0.05. 50 µL of the cultures were added to 96-well plates containing 50 µL of same medium with the compounds, serially diluted 1:2., for a final volume of 100 µL. The absorbance of the cultures during 4-5 days of incubation at 30^{o} C in an incubator was measured. Discontinuous readings were taken on the Biotek Synergy II Plate Reader at time points daily. Measured values were obtained and recorded with the Biotek Gen 5 2.03 program.

C. RESULTS AND DISCUSSION

Cloning of EKY3 containing pYES2.1HTopT718A

After the successful PCR mutagenesis reaction using the NEB kit, the amplicon corresponding to pYES-1 plasmid with HTOP1 gene carrying the T718A mutation was extracted using a DNA extraction kit (Zymo) according to manufacturer's instructions and transformed into NEB turbo *E. coli* competent cells (Figure 3.2.). The resulting transformants were cultured for plasmid extraction using miniprep kit (NEB) and the plasmids were commercially sequenced (Eurofins genomics). The sequence verified plasmid pYES2.1HTOPT718A was then transformed into competent *Saccharomyces cerevisiae* strain EKY3 with Top1 deletion mutation to abolish the yeast encoded topoisomerase IB activity.

a.



b.

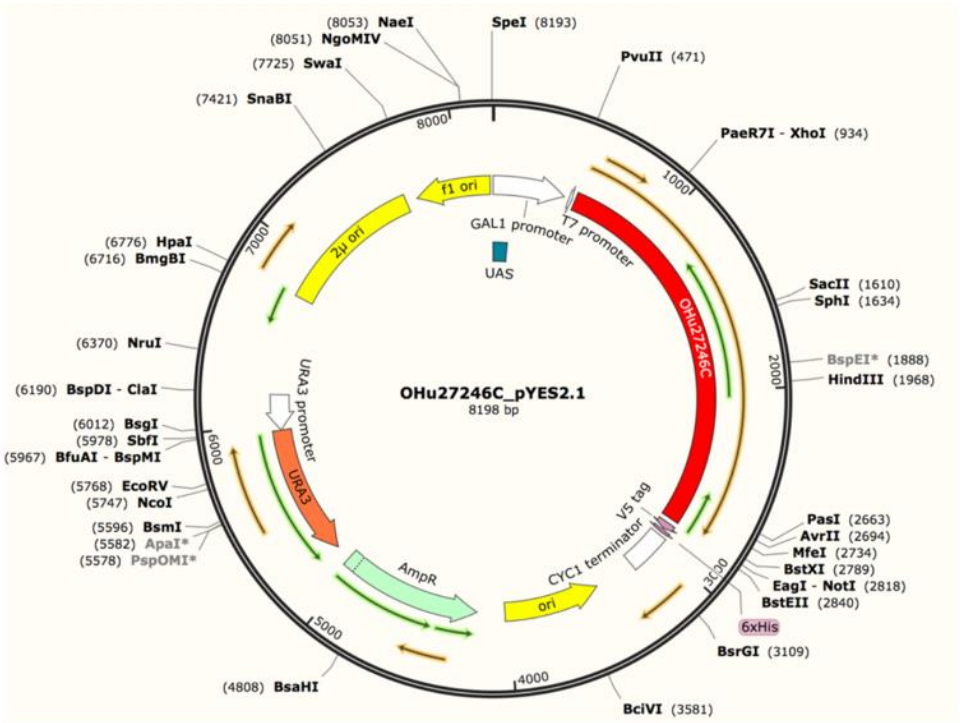


Figure 3.2. Site directed mutagenesis of PCR amplified pYES2.1HTop1T718A. (a) Well 1 consists of negative control (5 µL of 1 µL template pYES2.1 diluted in H_2O for a total volume of 25 µL). Well 2 and 3 contain Quick-load 1Kb DNA ladder and Top1 mutant clone, respectively. Reactions were terminated with 5x stop buffer. (b) Map of plasmid pYES2.1

Expression of HTop1T718A is lethal to yeast

10 µL of 10-fold serial dilutions of saturated cultures of the yeast transformants were spotted on SC-U plates. 2% galactose and 2% dextrose plates were utilized for comparison (figure 3.3.). Dilutions of the yeast expressing HTop1T718A were done in triplicate for each plate. Each row contains cultures taken from a single colony.

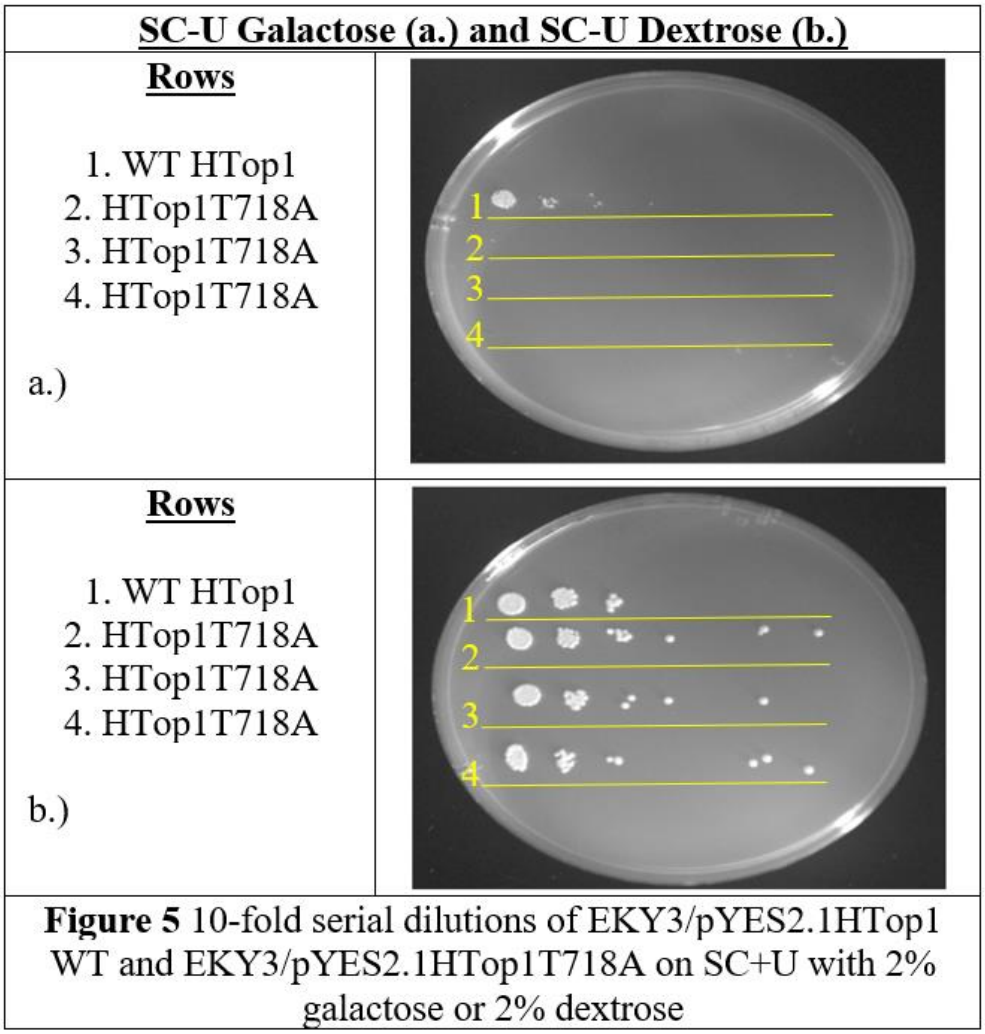


Figure 3.3. 10-fold serial dilutions of EKY3/pYES2.1HTop1 WT and EKY3/pYES2.1HTop1T718A on SC+U with 2% galactose or 2% dextrose

Dextrose suppresses HTop1 expression from the GAL1 promoter. Therefore, the strains expressing wild type and mutant HTop1 enzyme have similar growth in the presence of dextrose. The presence of galactose induces expression, causing cell death of strain overexpressing mutant enzyme that accumulates HTop1cc.

Effect of inhibitors on the growth of yeast expressing WT or mutant HTop1 in the presence of galactose and dextrose

Growth inhibition assay of EKY3-pYES2.1/HTop1 and EKY3-pYES2.1/HTop1T718A evaluates the ability of HTop1 to bind to DNA and form the HTop1cc in the presence of candidate inhibitors. Absorbance (OD_{600}) of EKY3-pYES2.1/HTop1 and EKY3-pYES2.1/HTop1T718A in the presence of an inhibitor compound were monitored at periodic time intervals. OD_{600} values were obtained after ~66 hours. The peak exponential growth phase was used for fold change of growth calculations. Fold change values are expressed as a ratio of the OD_{600} value of the yeast cells, at a given concentration of the compound, relative to the value in the absence of the compound. A fold change of 1 indicates no change in growth. An increase in growth compared to yeast in the absence of a compound has a value above 1 while a decrease in growth is below 1. CPT, a poison inhibitor of HTop1, was used as a control compound to evaluate and demonstrate the efficacy of the yeast growth assay. CPT causes an accumulation of Top1cc which leads to death. In the presence of galactose when WT HTop1 is expressed, EKY3-pYES2.1/HTop1 shows a strong dose dependent reduction in growth (figure 3.4a-c). There is no reduction in growth when the expression of WT HTop1 is repressed by dextrose. In the presence of galactose or dextrose, the effect of CPT on yeast cells expressing mutant HTop1T718A is minimal since the mutant enzyme already mimics the action of a poison inhibitor. A candidate compound suitable to treatment in sepsis should be able to bind mutant HTop1 and rescue growth inhibition, as indicated by a fold change in growth over 1.

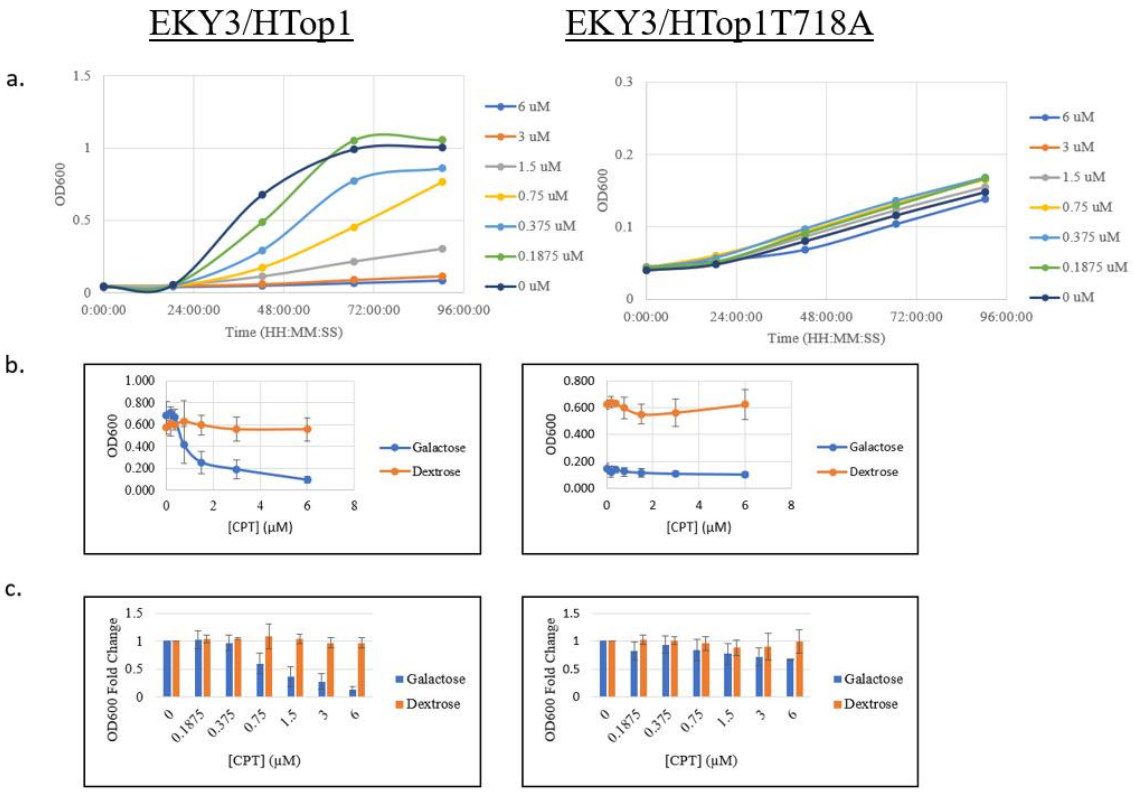


Figure 3.4. OD600 and fold change values of EKY3/HTop1 and EKY3/HTop1T718A under the presence of CPT (n=3) a.) Growth curves of EKY3 overexpressing HTop1 (left) and HTop1T718A (right) in galactose media. b.) OD600 values of EKY3-pYES2.1/HTop1 and EKY3-pYES2.1/HTop1T718A in the presence of 0-6 µM CPT at ~66 hours. The error bars report the deviation in values of multiple trials under identical conditions. c.) fold change values based on OD600 measurements at ~66 hours.

Inhibition of EKY3-pYES2.1HTop1 and EKY3-pYES2.1HTop1T718A in presence of inhibitor candidates

None of the compounds identified by *in-silico screening* rescued the growth of yeast when HTop1718A mutant is overexpressed in the presence of galactose (Figure 3.5a). This suggests that they do not affect the DNA binding and formation of lethal HTop1cc by the mutant HTop1718A, in agreement with the lack of inhibition of HTop1 relaxation activity observed in Chapter 2. Out of the *in-silico* compounds tested, NSC372499 and NSC158549 show nearly a twofold reduction in growth of the mutant strain but not the wild type from 0-100 µM (figure 3.5a). This in contrast to NSC379651 whereas no change in relative growth occurred for both strains. This suggests that NSC372499 and NSC158549 may render the yeast cell hypersensitive to mutant HTop1cc accumulation by inhibiting DNA repair functions in yeast cells.

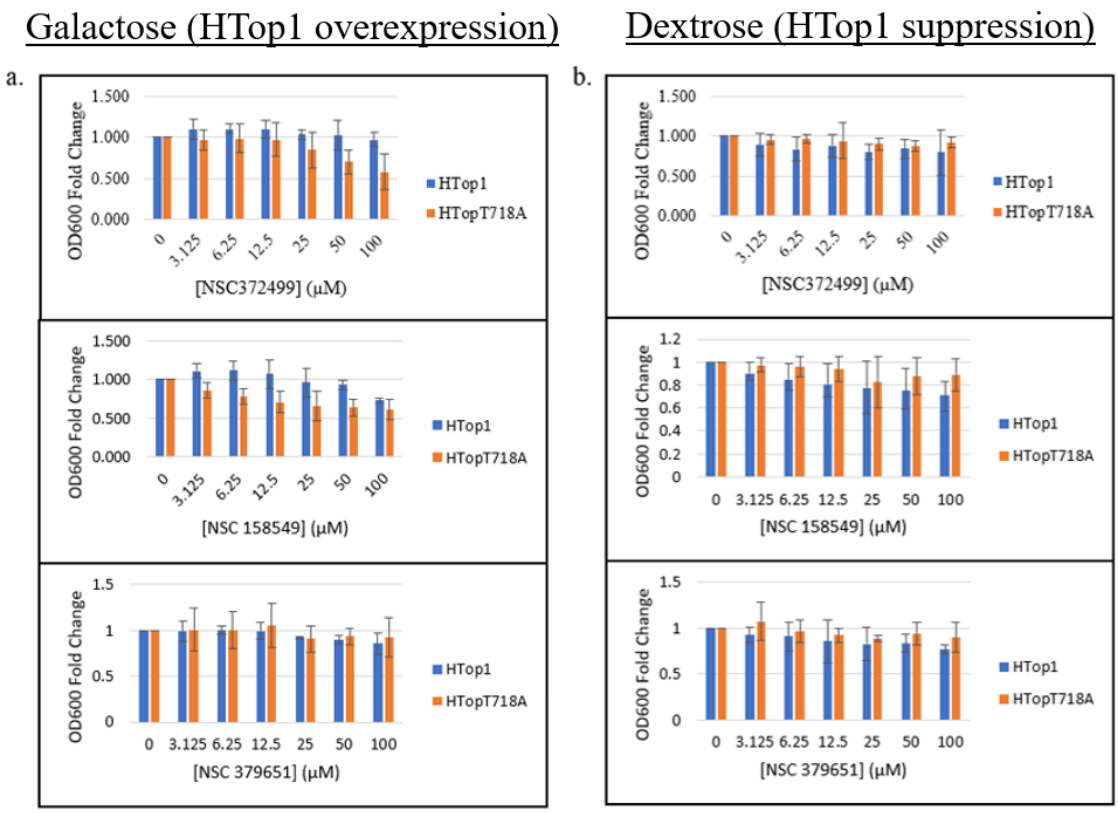


Figure 3.5. OD600 fold change of EKY3/HTop1 and EKY3/HTop1T718A in the presence of *in silico* screening compounds (n=4) a.) fold increase of OD600 values upon treatment with compounds with HTop1 overexpression induced by galactose in the media. b.) fold increase in OD600 values upon compound treatment in the absence of HTop1 overexpression with dextrose in the media. The error bars report the deviation in values of multiple trials under identical conditions.

A 1.2-fold increase in growth of the yeast strain overexpressing T718A mutant HTop1 when treated with delphinidin chloride ($P < 0.005$) and cyanidin chloride ($P < 0.005$) versus no increase in growth of yeast strain between the concentrations of 7.1825-62.5 µM overexpressing wild-type HTop1 (figure 3.6a.) serves as evidence for the ability of delphinidin chloride and cyanidin chloride to modulate HTop1cc accumulation by the T718 HTop1 mutant enzyme or protect the yeast cells from growth inhibition by Top1cc poison inhibition. Based on a previous study, delphinidin chloride ($P < 0.05$) was proposed as HTop1 cleavage inhibitors based on their ability to protect HT29 cells from the HTop1 poison inhibitor effects of CPT (Habermeyer et al., 2005). The data from the yeast growth inhibition assay shown here helps support the utility of this assay to study the mechanism of HTop1 inhibitors and identify catalytic inhibitors that decrease either DNA binding or cleavage by HTop1 instead of acting as HTop1 poison inhibitors.

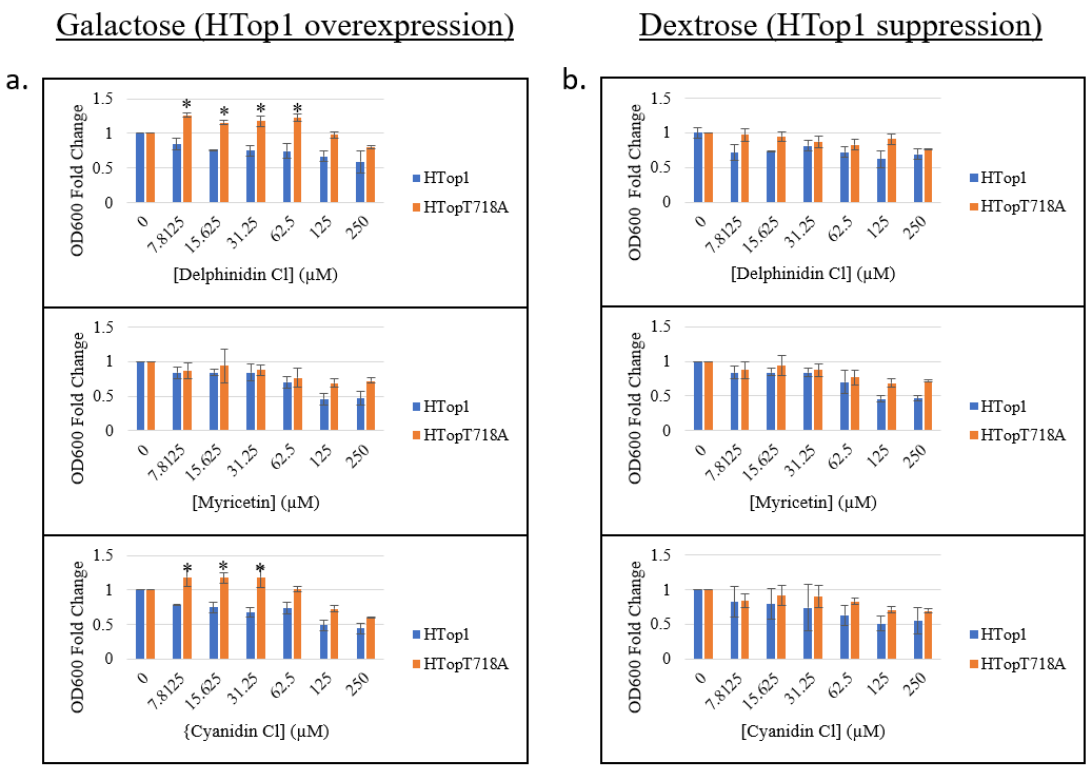


Figure 3.6. OD600 fold change of EKY3/HTop1 and EKY3/HTop1T718A in the presence of myricetin and anthocyanidins (n=3). a.) fold increase of OD600 values upon treatment with compounds with HTop1 overexpression induced by galactose in the media. b.) fold increase in OD600 values upon compound treatment in the absence of HTop1 overexpression with dextrose in the media. The error bars report the deviation in values of multiple trials under identical conditions. * Paired T-Test function in MS Excel was used to determine statistical significance of EKY3/HTop1T718A OD600 fold change with delphinidin chloride ($P < 0.005$) and cyanidin chloride ($P < 0.005$) treatment relative to no treatment.

Out of the other nine natural products with similar flavonoid structures as delphinidin chloride and cyanidin chloride that we tested in Chapter 2, myricetin was the only compound that showed inhibition of the HTop1 relaxation activity. In the yeast growth inhibition assay, in contrast to delphinidin chloride and cyanidin chloride, myricetin inhibits the growth of the yeast strain overexpressing wild-type or mutant HTop1 in the presence of galactose, and when HTop1 overexpression is suppressed in

dextrose. This suggests that the compound is interfering with growth of *S. cerevisiae* through another mechanism that may not be related to HTop1cc accumulation.
The quantities of Boehringer Ingelheim compounds we received were insufficient for us to conduct the yeast growth inhibition assays.

In *S. cerevisiae*, tyrosine phosphodiesterase I (TDP1) processes Top1cc formed between Top1 and DNA by generating free 3' ends from the Top1cc (Pommier et al., 2014). The role of TDP1 in Top1cc processing and DNA strand break repair was verified in TDP1 knockdown study (Pommier et al, 2014).

CHAPTER IV: CONCLUSIONS

A. SUMMARY

Efficacy of topoisomerase poisons, and drug-resistant cells that can overcome the trapped cleavage complex mechanism of topoisomerase poisons, can raise issues on top of the complications of sepsis if currently available topoisomerase poison inhibitors are to be used for treatment of sepsis that can cause death or serious organ damage during bacterial or viral infections (Gilbert et al. 2012). The identification of novel catalytic HTop1 inhibitors can add insight to the development of clinical drugs to treat sepsis in a non-cytotoxic way. The hit molecules identified from the *in-silico* screening assay, in addition to the other compounds tested, were examined for their ability to inhibit HTop1 catalytic activity using the relaxation assay. The study of the mechanism of inhibition will help establish structure-activity relationship (SAR). The designed approach used here in this study includes a novel screening assay utilizing yeast expressing recombinant wild-type and mutant HTop1 in addition to assay of relaxation inhibition of negatively supercoiled plasmid DNA by HTop1 to determine if the compounds affect the enzyme's catalytic activity by preventing DNA binding and formation of HTop1cc. The yeast growth inhibition is easier to conduct compared to DNA binding and cleavage assays that usually require the use of radioactive DNA substrates. The activity of a compound on purified Top1 activity does not always correspond to the effect of the compound yeast growth. Therefore, we can control conditions to determine if there are additional modes of action to account for the effect of the compounds on yeast growth.

In this study, we tested the yeast growth inhibition assay as a screening assay to identify HTop1 inhibitor compounds that can affect the ability of HTop1 to bind to DNA or cleave DNA to form the HTop1cc. This compliments the relaxation inhibition assay that informs on the compounds' ability to inhibit the overall relaxation of supercoiled DNA.

Compounds that showed inhibition of negative supercoiled DNA relaxation by HTop1 were tested further for their mechanism of action. As seen with the growth inhibition study, several compounds were unable to prevent the HTop1cc poison mode of inhibition in the mutant yeast, while compounds such as the anthocyanidins mitigated to some extent the mutant lethality. With growth inhibition as observed in the case with Myricetin present, it is uncertain if it is acting on other aspects of the yeast cell or the HTop1 protein itself. To verify that the latter is true, yeast strain transformed with an empty vector not expressing any HTop1 can be used to check if the same the same inhibition is observed. This will serve as a control.

Assaying cleavage activity of Top1 can help identify topoisomerase poisons based on measuring the amount of broken DNA formed by the drug and enzyme when the enzyme-DNA complex is denatured. Comet assay can be used to detect DNA damage, multiple ssDNA breaks are characteristic of a Top1 poison inhibitor. Taken together, the methods developed in the study can be used to access the mode of inhibition for structural analogs.

B. FUTURE STUDIES

Other natural products found in tropical plants or symbiotic fungi can be explored with the combination of the relaxation and yeast-based growth inhibition assay to identify additional scaffolds for novel catalytic inhibitors of HTop1. This would require fractionation of the plant or fungal extracts and characterization of the active compounds. Cytotoxicity of the HTop1 inhibitors and their ability to modulate the cytokine storm during sepsis will need to be evaluated for development of the catalytic HTop1 inhibitors into new treatment for sepsis.

LIST OF REFERENCES

Bei, L.; Dai, Y.; Liu, J.; Zhuge, Q.; Li, D.; The Effect of Dimethyl Sulfoxide on Supercoiled DNA Relaxation Catalyzed by Type 1 Topoisomerases. *Biomed Res. Int* **2015**, 320490.

Berger, J.M; Structure of DNA topoisomerases. *Biochim. Biophys. Acta. Gen. Subj.* **1998**, 1400, 13-18.

Bjornsti, M.A.; Benedetti, P.: Viglianti, G.A.; Wang, J.C.; Expression of Human DNA Topoisomerase I in Yeast Cells Lacking Yeast DNA Topoisomerase I: Restoration of Sensitivity of the Cells to the Antitumor Drug Camptothecin. *Cancer Res*. **1989**, *49*, 6318-6323.

Carson, M.; RIBBONS 2.0. *J Appl. Cryst.* **1991**, 24(5): 958-961.

Chen, L., Sabo, J.P.; Philip, E.; Rowland, L.; Mao, Y.; Latli, B.; Ramsden, D.; Mandarino, D.A; Sane, R.S. Mass Balance, Metabolite Profile, and In Vitro-In Vivo Comparison Clearance Pathways of Deleobuvir, a Hepatitis C Virus Polymerase Inhibitor. *Antimicrob. Agents Chemother.* 2015, 59(1)

Chrencik, J.E, Staker, B.L, Burgin, A.B., Pourquier, P., Pommier, Y., Stewart, L., Redinbo, M.R. Mechanisms of Camptothecin Resistance by Human Topoisomerase I Mutations. *J Mol. Biol.* **2004**, 339, 773-784.

Chowdhury, A.R.; Sharma, S.; Mandal, S.; Goswami, A.; Mukhopadhyay, S.; Majumdee, H.K.; Luteolin, an emerging anti-cancer flavonoid, poisons eukaryotic DNA topoisomerase I. *Biochem J.* **2002**, 366, 653-661

Constantinou, A.; Mehta, R.; Runyan, C.; Flavonoids as DNA Topoisomerase Antagonists and poisons: structure-activity relationships. *J. Nat. Prod.* **1995**, 58(2): 217-225.

Corbett, K.D; Berger, J.M.; Structure, Molecular Mechanisms, And Evolutionary Relationships in DNA Topoisomerases. *Annu. Rev. Biophys. Biomol.* **2004**, 33, 95-118.

Corless, S.; Gilbert, N.; Investigating DNA supercoiling in eukaryotic genomes. *Brief Funct. Geonomics* **2017**, 16(6): 379-389.

Cretaio, E.; Pattarello, L.; Fontebasso, Y.; Benedetti, P.; Losasso, C. Human DNA Topoisomerase IB: Structure and Functions. *Ital. J. Biochem.* **2007**, 56(2): 91-102.

Deweese, J.E.; Osheroff, M.A.; Osheroff, N.; DNA Topology and Topoisomerases. *BAMBED* **2009**, 37(1): 2-10.

Esselen, M.; Fritz, J.; Hutter, M.; Marko, D.; Delphinidin Modulates the DNA-Damaging Properties of Topoisomerase II Poisons. *Chem. Res. Toxicol.* **2009**, 22(3): 554-564.

Esselen, M.; Fritz, J.; Hutter, M.; Teller, N.; Baechler, S.; Boettler, U.; Marczylo, T.H.; Gescher, A.J.; Marko, D.; Anthocyanin-rich extracts suppress the DNA-damaging effects of topoisomerase poisons in human colon cancer cells. *Mol. Nutr. Food Res.* **2011** *55*, S143-S153.

Gilbert, D. C.; Chalmers, A. J.; El-Khamisy, S.F; Topoisomerase I inhibition in colorectal cancer: biomarkers and therapeutic targets. *Br. J. Cancer* **2012,** 106(1): 18-24.

Goldstein, M.; Kastan, M.B. The DNA Damage Response: Implications for Tumor Responses to Radiation and Chemotherapy. *Annu. Rev. Med.* **2015**, 66: 129-43.

Goto, T.;Wang, J.C.; Cloning of yeast TOP1, the gene encoding DNA topoisomerase I, and construction of mutants defective in both DNA topoisomerase I and DNA topoisomerase II. *Proc. Natl. Acad. Sci*. **1985** 82(21): 7178-7182.

Gupta, M.; Fujimori, A.; Pommier; Y.; Eukaryotic DNA topoisomerases I. *Biochim. Biophys. Acta* **1995**, 1262, 1-14

Fiorani, P.; Amatruda, J.F.; Silvestri, A.; Butler, R.H; Bjornsti, M.A.; Benedetti, P.; Domain Interactions Affecting Human DNA Topoisomerase I Catalysis and Camptothecin Sensitivity. *Mol. Pharacol.* **1999**, *56*, 1105-1115.

Habermeyer, M.; Fritz, J.; Barthelmes, H.U.; Christensen, M.O.; Larsen, M.K.; Boege, F.; Marko, D. Anthocyanidins Modulate the Activity of Hyman DNA Topoisomerases I and II and Affect Cellular DNA Integrity. *Chem Res. Toxicol* **2005**, 18, 1395-1404.

Ho, J.S.Y. et al.; Topoisomerase I inhibition therapy protects against SARS-CoV-2-induced inflammation and death in animal models. *bioRxiv* 2020, doi: 10.1101/2020.12.01.404483.

Khoo, H.E; Azlan, A.; Tang, S.T.; Lim, S.M.; Anthocyanidins and anthocyanins: colored pigments as food, pharmaceutical ingredients, and the potential health benefits. *Food Nutr. Res.* **2007**, 61, 1361779.

Koster, D.A.; Palle, K.; Bot, E.S.M.; Bjornsti, M.; Dekker, N.H.; Antitumor drugs impede DNA uncoiling by topoisomerase I. *Nature* **2007**, 448.

Krough, B.O.; Shuman, S.; Catalytic Mechanism of DNA Topoisomerase IB. *Mol. Cell* **2000**, 5, 1035-1041.

Kumar, S.; Pandey, A.K.; Chemistry and Biological Activities of Flavonoids: An Overview. *Sci.World J.* **2013**, 162750.

Lopez-Lazaro,M.; Willmore,E.; Austin, C.A. The dietary flavonoids myricetin and filet in act as dual inhibitors of DNA topoisomerases I and II in cells. *Mutat. Res.* **2010**, 696(1): 41-47.

Nitiss, J.L.; Soans, E.; Rogojina, A.; Seth, A.; Mishina, M.; Topoisomerase Assays. *Curr. Protoc. Pharamcol.* **2012**, 57.

Pommier, Y.; Drugging Topoisomerases: Lessons and Challenges. ACS Chemical Biology. *ACS Chem. Biol.* **2013**, *8*, 82-95.

Pommier, Y.; Huang, S.N.; Gao, R.; Das, B.B.; Murai, J.; Marchand, C.; Tyrosyl-DNA-phosphodiesterases (TDP1 and TDP2). *DNA Repair (Amst)* **2014**, 19, 114-120

Rialdi, A. et al. Topoisomerase 1 inhibition suppresses inflammatory genes and protects from death by inflammation. *Science.* **2016**, *352*(6289).

Redinbo, M.R; Stewart, L.; Kuhn, P.; Champoux, J.J.; Crystal Structures of Human Topoisomerase I in Covalent and Noncovalent Complexes with DNA. *Science* **1998**, 279(5356): 1504-1513.

Sandhaus, S.; Annamalai, T.; Welmaker, G.; Houghten, R.A.; Paz, C.; Garcia, P.K.; Andres, A.; Narula, G.; Rodrigues Felix, C.; Geden, S.; Netherton, M.; Gupta, R.; Rohde, K.H.; Giulianotti, M.A.; Tse-Dinh, Y.C.; Small-Molecule Inhibitors Targeting Topoisomerase I as Novel Antituberculosis Agents. *Antimicrob. Agents Chemother*. **2016**, *60*, 7.

Sarafianos, S.G.; Marchand, B.; Das, K.; Himmel, D.M.; Parniak, M.A; Hughes, S.H.; Arnold, E. Structure and Function of HIV-1 Reverse Transcriptase: Molecular Mechanisms of Polymerization and Inhibition. *J. Mol. Biol*. **2009**, 385, 693-713.

Schoeffler, A., & Berger, J.; DNA topoisomerases: Harnessing and constraining energy to govern chromosome topology. *Q. Rev. Biophys.* **2008** 41(1), 41-101.

Soren, B.C; Dasari, J.B.; Ottaviani, A.; Iacovelli, F.; Fiorani, P.; Topoisomerase IB: a relaxing enzyme for stressed DNA. *Cancer Drug Resist.* **2020**, 3: 18-25.

Tiwari, P.B.; Chapagain, P.P.; Seddek, A.; Annamalai, T.; Üren, A.; Tse-Dinh, Y.C.,; Covalent Complex of DNA and Bacterial Topoisomerase: Implications in Antibacterial Drug Development. *: ChemMedChem* **2020,** 10.1002/cmdc.201900721

Xin, L., Liu, L., Shao, C., Yu, R., Chen, F., Yue, S., Wang, M., Guo, Z., Fan, Y., Guan, H., Wang, C. (2017) Discovery of DNA Topoisomerase I Inhibitors with Low-Cytotoxicity Based on Virtual Screening from Natural Products. *Mar. Drugs* **2017**, *15*, 217.

Wang, J.C.; Cellular Roles of DNA Topoisomerases: A Molecular Perspective. *Nat. Rev. Mol. Cell Biol.* **2002** 3(6): 430-40.

Webb, M.R.; Ebeler, S.E; Comparative analysis of topoisomerase IB inhibition and DNA intercalation by flavonoids and similar compounds. *Biochem. J.* **2004**, 384, 524-41.

Webb, M.R; Min, K., Ebeler, S.E. Anthocyanin Interactions with DNA: Intercalation, Topoisomerase I Inhibition and Oxidative Reactions. *J Food Biochem.* **2008**, 32(5): 576-596.

Yu, Q.; Chen, Y.; Yang; H.; Zhang, H.; Agama, K.; Pommier, Y.; An, L.; The anti-tumor activity of CYB-L10, a human topoisomerase IB catalytic inhibitor. *J. Enzyme Inhib. Med. Chem.* **2019**, 34(1), 818-822.