



เลขที่เอกสาร: 61120115310198000

สวพ-ว-4(1)

ใบนำส่งรายงานวิจัย

เลขที่รับ(สวพ).....

วันที่รับ.....

ขอส่งรายงานวิจัย มก. ประจำปีงบประมาณ (1) 2554

(2) ลักษณะโครงการ เป็นโครงการวิจัยเดี่ยว รหัส ว-ท(ด)49.53 ชื่อโครงการ การวิเคราะห์แต่ละส่วนของ เอนไซม์ Topoisomerase II alpha และ beta จากมนุษย์ต่อการจับกับกับสารกลุ่ม Rhinacathins และ Napthoquinone esters

(3) หัวหน้าโครงการ ผศ.นลวัฒน์ บุญญาลัย

(4) หน่วยงาน ภาควิชาชีวเคมี คณะวิทยาศาสตร์ บางเขน

(5) ประเภทโครงการวิจัย โครงการวิจัย 3 สาขา โครงการวิจัยสาขาวิทยาศาสตร์และเทคโนโลยี

(6) รายงานที่ส่ง รายงานผลการวิจัยฉบับสมบูรณ์ (ฉบับจริง) จำนวน 8 ชุด พร้อม CD/Diskette 2 แผ่น

(7) การเผยแพร่ผลงานวิจัย ประสงค์ให้ สวพ. เผยแพร่ได้

ลงชื่อ.....

(ผศ.นลวัฒน์ บุญญาลัย)

หัวหน้าโครงการ

14 พ.ย. 2556

ใบรับรายงานวิจัย

เลขที่รับ(สวพ).....

วันที่รับ.....

ขอส่งรายงานวิจัย มก. ประจำปีงบประมาณ (1) 2554

(2) ลักษณะโครงการ เป็นโครงการวิจัยเดี่ยว รหัส ว-ท(ด)49.53 ชื่อโครงการ การวิเคราะห์แต่ละส่วนของ เอนไซม์ Topoisomerase II alpha และ beta จากมนุษย์ต่อการจับกับสารกลุ่ม Rhinacathins และ Napthoquinone esters

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(7) การเผยแพร่ผลงานวิจัย ประสงค์ให้ สวพ. เผยแพร่ได้

ลงชื่อ.....

(เจ้าหน้าที่สถาบันวิจัยและพัฒนา)

...../...../.....



รายงานผลการวิจัยฉบับสมบูรณ์
ทุนอุดหนุนวิจัย มก.ปีงบประมาณ 2554

รหัสโครงการวิจัย ว-ท(ด)49.53

การวิเคราะห์แต่ละส่วนของเอนไซม์ Topoisomerase II alpha และ beta จากมนุษย์ต่อ
การจับกับสารกลุ่ม Rhinacathins และ Napthoquinone esters

Domain analysis of Human Topoisomerase II alpha and beta towards the binding of
Rhinacanthins and Napthoquinone esters

หัวหน้าโครงการ ผศ.นลวัฒน์ บุญญาลัย

หน่วยงานต้นสังกัด ภาควิชาชีวเคมี คณะวิทยาศาสตร์ บางเขน

หน่วยงานหลัก ภาควิชาชีวเคมี คณะวิทยาศาสตร์ บางเขน

แหล่งทุน : ทุนอุดหนุนวิจัย มก.

สถาบันวิจัยและพัฒนาแห่งมหาวิทยาลัยเกษตรศาสตร์

แบบรายงานผลการวิจัยฉบับสมบูรณ์
โครงการวิจัย (Project)
โครงการวิจัยทุนอุดหนุนวิจัย มก. ปีงบประมาณ 2554

ส่วนที่ 1 ข้อมูลโครงการวิจัย

- 1.1 รหัส ว-ท(ด)49.53 ชื่อโครงการวิจัย การวิเคราะห์แต่ละส่วนของเอนไซม์ Topoisomerase II alpha และ beta จากมนุษย์ต่อการจับกับสารกลุ่ม Rhinacathins และ Naphthoquinone esters
- 1.2 ลักษณะโครงการ เป็นโครงการวิจัยเดี่ยว
- 1.3 ชื่อหัวหน้าโครงการ ผศ.นลวัฒน์ บุญญาลัย
- 1.4 หน่วยงานต้นสังกัด ภาควิชาชีวเคมี คณะวิทยาศาสตร์ บางเขน
 หน่วยงานหลัก ภาควิชาชีวเคมี คณะวิทยาศาสตร์ บางเขน
- 1.5 ประเภทโครงการ โครงการวิจัย 3 สาขา โครงการวิจัยสาขาวิทยาศาสตร์และเทคโนโลยี
- 1.6 ระยะเวลาดำเนินงานวิจัยตลอดโครงการ 2 ปี ตั้งแต่ปีงบประมาณ 2553 ถึงปีงบประมาณ 2554
- 1.7 สถานที่ดำเนินงานวิจัย/เก็บข้อมูล
 - ห้องปฏิบัติการภาควิชาชีวเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยเกษตรศาสตร์
 เลขที่ 50 ถนนพหลโยธิน แขวงลาดยาว เขตจตุจักร
 กรุงเทพฯ 10900 โทรศัพท์ 02-562-5555 ต่อ 2045 โทรสาร 02-5614627
- 1.8 งบประมาณรวมตลอดโครงการ 380,000.00 บาท ประกอบด้วย
 - ปีงบประมาณ 2553 ได้รับ 200,000.00 บาท
 - ปีงบประมาณ 2554 ได้รับ 180,000.00 บาท
- 1.9 วัตถุประสงค์โครงการวิจัย
 1. ศึกษาถึงความสำคัญของแต่ละ domain ของเอนไซม์ Topoisomerase II alpha และ beta กับสารกลุ่ม rhinacanthin และ naphthoquinone ester โดยเทคนิคทางชีวเคมี
 2. ศึกษากรดอะมิโนที่สำคัญต่อการจับกันอย่างจำเพาะระหว่างเอนไซม์ Topoisomerase II ทั้งสอง isoform กับสารกลุ่ม rhinacanthin และ naphthoquinone ester โดยเทคนิคทางชีวเคมี เช่น site-directed mutagenesis, Fluorescent spectroscopy และ ATP coupling assay
- 1.10 เป้าหมายผลงานวิจัยตลอดโครงการ

ปีงบประมาณ	เดือนที่	ผลงานวิจัยที่คาดว่าจะได้
2554	1-6	ศึกษากิจกรรมของเอนไซม์ ATPase TopoII alpha และ beta
	7-12	ศึกษาการยับยั้งกิจกรรมของเอนไซม์ ATPase TopoII alpha และ beta กับสารกลุ่ม Rhinacanthin derivatives

2553	1-6	ทำการโคลนนิ่งส่วน ATPase domain ของเอนไซม์ human TopoII alpha และ beta
	7-12	ผลิตและทำบริสุทธิ์เอนไซม์ ATPase TopoII alpha และ beta

1.11 สรุปผลการดำเนินงานวิจัยตลอดโครงการ

- วัตถุประสงค์ (ตามแผน)

1. Cloning, expression, purification and characterization of ATPase domain of human Topoisomerase II alpha and beta

2. Molecular docking and Site-directed mutagenesis

- เป้าหมาย/ผลที่คาดหวัง (ตามแผน)

1. Pure and active enzyme with kinetic parameters

2. Reveal the binding site of Rhinacanthins on human TopoII ATPase domain as well as the important amino acid residues.

- ผลการดำเนินงาน (ปฏิบัติได้จริง)

1. Enzyme ATPase_TopoII_alpha can be overexpressed and purified to high yield but the ATPase_TopoII_beta was expressed as the inclusion bodies. Enzyme kinetics and inhibition study of ATPase_TopoII_alpha was carried out.

2. Molecular docking of ATPase_TopoII_alpha with some selected naphthoquinone derivative was performed. However, the site-directed mutagenesis was not carried out.

1.12 ผลการดำเนินงานวิจัยเป็นไปตามแผนหรือไม่ อย่างไร

- ไม่เป็นไปตามแผน

The mutagenesis experiment has not been established yet but the research will continue working on the mutation of ATPase_TopoII_alpha as to proof the result from molecular docking.

1.13 ปัญหา อุปสรรคในการดำเนินงาน และแนวทางแก้ไข

- ไม่มีปัญหาและอุปสรรค

1.14 สรุปผลการดำเนินงานตามวัตถุประสงค์

- บรรลุบางส่วน (ร้อยละ 80) เหตุผล The mutagenesis work will be carried out in the future.

1.15 ผลผลิต/สิ่งที่ได้จากการวิจัย (Outputs)

- หัวเรื่องวิทยานิพนธ์

INHIBITORY MECHANISM STUDY OF TOPOISOMERASE II BY RHINACANTHINS AS POTENTIAL ANTICANCER DRUGS

1.16 จุดเด่นของผลงานวิจัย / ผลผลิต / สิ่งที่ได้จากการวิจัย (outputs)

- สร้างองค์ความรู้ใหม่/นวัตกรรมที่ทันสมัย

งานวิจัยนี้ได้แสดงให้เห็นถึงความสำคัญของสารในกลุ่ม Rhinacanthin และ naphthoquinone derivatives ซึ่งสมควรได้รับการทำวิจัยเพิ่มเติม และ พัฒนาให้ได้เป็นยาต้านโรคมะเร็งจากสมุนไพรไทย

1.17 การนำผลการวิจัยไปใช้ประโยชน์ (Outcomes)

1. การนำผลการวิจัยไปเผยแพร่/ถ่ายทอด

1.1 วารสารวิชาการระดับชาติ/วารสารวิชาการระดับนานาชาติ 2 เรื่อง

ตีพิมพ์ในวารสารวิชาการระดับนานาชาติ

- ผู้แต่ง : Pradidphol N, Kongkathip N, Sittikul P, Boonyalai N, Kongkathip B.

- ชื่อเรื่อง : First synthesis and anticancer activity of novel naphthoquinone amides ชื่อวารสาร

: European journal of medicinal chemistry

- ปีที่ตีพิมพ์ : 2555 เดือน: มกราคม ถึง มกราคม เล่มที่ : 49 ฉบับที่ : 0 หน้า : 253 ถึง 270

ตีพิมพ์ในวารสารวิชาการระดับนานาชาติ

- ผู้แต่ง : Boonyalai N, Sittikul P, Pradidphol N, Kongkathip N

- ชื่อเรื่อง : Biophysical and molecular docking studies of naphthoquinone derivatives on the

ATPase domain of human topoisomerase II ชื่อวารสาร : Biomedicine & pharmacotherapy

- ปีที่ตีพิมพ์ : 2556 เดือน: มกราคม ถึง มกราคม เล่มที่ : 67 ฉบับที่ : 0 หน้า : 122 ถึง 128

1.2 นำเสนอในการประชุม/สัมมนาระดับชาติและนานาชาติ 1 เรื่อง

นำเสนอในการประชุม/สัมมนาระดับนานาชาติ

- ลักษณะเอกสาร/รูปแบบการนำเสนอ : ไม่มีการตีพิมพ์/ภาคโปสเตอร์

- ชื่อผู้เสนอผลงาน : Sittikul, P, Kongkathip, N and Boonyalai, N

- ชื่อเรื่อง : Inhibitory mechanism study of human topoisomerase II by rhinacanthins as potential anticancer drugs

- ชื่อการประชุมสัมมนา : The 3rd International Conference on Biochemistry and Molecular Biology Conference

- วัน/เดือน/ปี : จาก 6 เม.ย. 2554 ถึง 8 เม.ย. 2554

- สถานที่/เมือง/ประเทศ : Chiang Mai, Thailand

- หน้า : 183 ถึง 183

1.3 เผยแพร่ผลงานในรูปแบบการจัดนิทรรศการ

-

1.4 บทความ

-

1.5 จัดอบรมถ่ายทอด

-
- 1.6 นำเสนอทางสื่อผสม
-
- 1.7 ภาครัฐนำไปใช้กำหนดแผน/นโยบาย
-
- 1.9 อื่นๆ
-
- 2. เป้าหมายการนำผลลัพธ์ / ผลสำเร็จที่ได้ / หรือคาดว่าจะได้จากการวิจัยไปใช้ประโยชน์
 1. ด้านการศึกษา/เสริมการเรียนการสอน
 - ใช้ผลการวิจัยในการเรียนการสอนเนื่องจากเอนไซม์ Topoisomerase เป็นเอนไซม์ที่สำคัญในสิ่งมีชีวิต
- 1.18 ผลกระทบ (Impact) ที่เกิดจากการนำผลการวิจัยไปใช้ สอดคล้องกับยุทธศาสตร์ด้านใด
 - ยุทธศาสตร์การบริหารราชการแผ่นดิน (พ.ศ.2548 - 2551)
 - 1 . ยุทธศาสตร์การพัฒนามาบนฐานความหลากหลายทางชีวภาพและการสร้างความมั่นคงของฐานทรัพยากรและสิ่งแวดล้อม

เป้าประสงค์ การพัฒนาคุณค่าความหลากหลายทางชีวภาพ และภูมิปัญญาท้องถิ่น
 2. ยุทธศาสตร์การพัฒนาคูณภาพคนและสังคมไทยสู่สังคมแห่งภูมิปัญญาและการเรียนรู้

เป้าประสงค์ การเสริมสร้างสุขภาพของคนไทยให้มีสุขภาพแข็งแรงทั้งกายและใจ มีความสัมพันธ์ทางสังคมและอยู่ในสภาพแวดล้อมที่น่าอยู่
 3. ยุทธศาสตร์การสร้างความเข้มแข็งของชุมชนและสังคมให้เป็นรากฐานที่มั่นคงของประเทศ

เป้าประสงค์ การเสริมสร้างศักยภาพของชุมชน ในการอยู่ร่วมกันกับทรัพยากรธรรมชาติ และสิ่งแวดล้อมอย่างสันติและเกื้อกูล
 4. ยุทธศาสตร์การปรับโครงสร้างเศรษฐกิจให้สมดุลและยั่งยืน

เป้าประสงค์ การปรับโครงสร้างการผลิตเพื่อเพิ่มผลิตภาพ และคุณค่าของสินค้าและบริการบนฐานความรู้และความเป็นไทย
 - 5 . ยุทธศาสตร์การเสริมสร้างธรรมาภิบาลในการบริหารจัดการประเทศ มุ่งเสริมสร้างความเป็นธรรมในสังคมอย่างยั่งยืน

เป้าประสงค์ เสริมสร้างความเข้มแข็งของภาคประชาชน ให้สามารถเข้าร่วมในการบริหารจัดการประเทศ
 - นโยบายและยุทธศาสตร์การวิจัยของชาติ(พ.ศ.2551 - 2553)

ยุทธศาสตร์การวิจัยที่ 5 การบริหารจัดการความรู้ ผลงานวิจัย นวัตกรรม สิ่งประดิษฐ์ ทรัพยากร และภูมิปัญญาของประเทศ ส่งการใช้ประโยชน์เชิงพาณิชย์และสาธารณะ ด้วยยุทธวิธีที่เหมาะสม

กลยุทธ์การวิจัยที่ 1 วิจัยเพื่อพัฒนาระบบบริหารงานวิจัยของประเทศ ตลอดจนการบริหารจัดการความรู้

ผลงานวิจัย นวัตกรรม สิ่งประดิษฐ์ ทรัพยากร และภูมิปัญญาของประเทศ และจากต่างประเทศสู่การใช้ประโยชน์เชิงพาณิชย์และสาธารณะ

แผนงานวิจัยที่ 4 การวิจัยเกี่ยวกับการเข้าถึงทรัพยากรทางวิทยาศาสตร์และเทคโนโลยี และวิทยาการสาขาต่าง ๆ ตลอดจนองค์ความรู้ในประเทศและจากต่างประเทศ

1.19 การรับความคุ้มครองทรัพย์สินทางปัญญา

-

1.20 การได้รับรางวัล

-

1.21 งานที่จะทำต่อไป

- สำหรับงานวิจัย ที่อาจจะทำต่อไปคือการศึกษเปรียบเทียบการยับยั้งของสาร 4 กลุ่มคือ naphthoquinone aromatic esters/amides และ naphthoquinone aliphatic esters/amides ต่อเอนไซม์ human Topoisomerase II alpha_ATPase และ human Topoisomerase II beta_ATPase

1.22 คำชี้แจงเพิ่มเติม

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1.23 ได้แนบรายงานผลการวิจัยฉบับสมบูรณ์ของโครงการ (Project) ตามหัวข้อในส่วนที่ 2 มาด้วยแล้ว

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(ผศ.นลวัฒน์ บุญญาด้อย)

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ส่วนที่ 2

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การวิเคราะห์แต่ละส่วนของเอนไซม์ Topoisomerase II alpha และ beta จากมนุษย์ต่อการจับกับสารกลุ่ม
Rhinacathins และ Naphthoquinone esters

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สารอนุพันธ์ของ naphthoquinone เช่น rhinacanthin พบว่ามีความสามารถในการใช้เป็นยา
รักษาโรคมะเร็ง ซึ่งตัวเอนไซม์เป้าหมายของสารกลุ่มนี้ก็คือ hTopoll โครงสร้างของเอนไซม์ hTopoll
ประกอบด้วยส่วน ATPase domain และ ส่วน DNA binding domain ยาหลายชนิดอาจจับได้เพียง
บริเวณเดียวหรือทั้งสองบริเวณ จากการศึกษาเราพบว่า สารกลุ่ม naphthoquinone esters และ
amides สามารถยับยั้งกิจกรรมของเอนไซม์ hTopoll alpha ได้โดยพบว่าเมื่อเพิ่มความเข้มข้นของ
ATP การยับยั้งของสารกลุ่มนี้ต่อเอนไซม์จะลดลง ดังนั้นเพื่อที่จะศึกษาลักษณะการยับยั้งของ
สารกลุ่มนี้ต่อเอนไซม์ hTopoll ผู้วิจัยจึงได้ทำการผลิตรีคอมบิแนนท์โปรตีนของ hTopoll ATPase
domain เนื่องจากในมนุษย์จะพบ hTopoll อยู่สองฟอร์มคือ alpha และ beta ดังนั้นผู้วิจัยจึงทำการ
ผลิตทั้งสองโปรตีนโดยใช้เซลล์ของ E.coli เป็นเจ้าของบ้านในการผลิตโปรตีน จากการทดลองพบว่า
เฉพาะ hTopoll alpha จะพบว่ายูอยู่ในส่วนของ soluble fraction ส่วน hTopoll beta จะพบอยู่ใน
ส่วนของ inclusion bodies ดังนั้นผู้วิจัยจึงทำการทดลองต่อเฉพาะ hTopoll alpha-ATPase เท่านั้น
และพบว่า กิจกรรมของ hTopoll alpha-ATPase จะลดลงเมื่อมีสาร naphthoquinone derivatives
อยู่ นอกจากนี้ผู้วิจัยได้ทำการทดลอง molecular docking เพื่อจะแสดงถึงลักษณะการจับการของ
สารกลุ่มนี้ต่อ hTopoll alpha-ATPase จากการทดลองพบว่าสารในกลุ่ม naphthoquinone
derivatives จับในบริเวณเดียวกับสาร ATP ซึ่งผลจากการทดลอง docking นั้นมีความสอดคล้องกับ
การทดลองการจับกันโดยติดตามด้วยเครื่องฟลูออเรสเซนซ์ จากการทดลองทั้งหมดทำให้เราสามารถ
นำความรู้ที่ได้ไปพัฒนาและออกแบบสาร rhinacanthin ตัวอื่น ๆ ได้ในอนาคตเพื่อใช้เป็นยารักษาโรคมะเร็ง

คำสำคัญ : เอนไซม์โทโปไอโซเมอเรส II , สารไรนาแคนทิน , สารเนปโทควินโนนเอไมด์ , สารเนปโทควินโนนเอสเทอร์

ABSTRACT

Numerous naphthoquinone derivatives such as rhinacanthins function as anticancer drugs, which target hTopoll. The structure of hTopoll contains both an ATPase domain and

a DNA binding domain. Several drugs bind to either one or both of these domains, thus modifying the activity of hTopoII. The naphthoquinone esters and amides used in this study showed that their hTopoII alpha inhibitory activity was inversely proportional to ATP concentration. In order to better characterize the inhibitory action of these compounds, sufficient quantities of soluble functional hTopoII ATPase domain were required. Therefore both the alpha and beta isoforms of the hTopoII ATPase domain were over-expressed in E. coli. The hTopoII alpha-ATPase activity was reduced in the presence of naphthoquinone derivatives. Additionally, a molecular docking study revealed that the selected naphthoquinone ester and amide bind to the ATP-binding domain of hTopoII alpha. Collectively, the results here provide for the first time a novel insight into the interaction between naphthoquinone esters and amides, and the ATP binding domain of hTopoII alpha. The further elucidation of the mechanism of action of the naphthoquinone esters and amides inhibitory activity is essential. In the solution, the quenching in fluorescence was observed and the K_d values can be determined. These results will allow the structure-based designing of novel Rhinacanthins in the foreseeable future.

Key words : Topoisomerase II , Rhinacanthins , Naphthoquinone amides , Naphthoquinone esters

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คำนำ

รายงานเล่มนี้เป็นรายงานประกอบงานวิจัยในหัวข้อ การวิเคราะห์แต่ละส่วนของเอนไซม์ Topoisomerase II alpha และ beta จากมนุษย์ต่อการจับกับสารกลุ่ม Rhinacathins และ Naphthoquinone esters ซึ่งได้รับการสนับสนุนเงินวิจัยจาก โครงการวิจัยทุนอุดหนุนวิจัย มก. ปีงบประมาณ 2554 รหัส ว-ท(ด)49.53 โดยรายงานนี้ได้กล่าวถึง การศึกษากลไกการยับยั้ง ATPase domain ของ human Topoisomerase II เพื่อใช้เป็นเป้าหมายในการศึกษาการยับยั้งกิจกรรมของเอนไซม์โดยสารในกลุ่ม rhinacanthin ได้แก่ naphthoquinone esters และ amides ทางผู้วิจัยหวังว่ารายงานฉบับนี้จะเป็นประโยชน์ต่อผู้สนใจในการวิจัยเกี่ยวกับการต่อต้านโรคมะเร็ง

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1. DNA replication

DNA in all species ranging from bacteria to humans is globally underwound (*i.e.* negatively supercoiled). This underwinding makes it easier to separate complementary DNA strands from one another and greatly facilitates initiation of replication and the assembly of replication forks. Once the fork begins to travel along the DNA template, the deleterious effects of topology manifest themselves (Figure. 1). As can be seen in Figure 1A, DNA, since helicases separate the two strands of the double helix, but do not unwind them, fork movement results in acute overwinding (*i.e.* positive supercoiling) of the DNA ahead of the replication machinery (Figure. 1B). This overwinding has two major consequences. Firstly, it increases the difficulty of separating duplex DNA into individual strands. Therefore, the accumulation of positive supercoils presents a formidable block to fork movement. Secondly, DNA overwinding ahead of the fork leads to a compensatory underwinding behind the replication machinery. (McClendon K. A. *et.al*, 2005)

In DNA replication, any rotation of the replication fork that does occur leads to an intertwining of the daughter chromosome), which then result in catenanes. Once the replication is completed these catenanes are parental double – helical turns that have not been removed by relaxation of supercoils ahead of the fork and persist after the replication finishes. Catenanes, therefore, must be removed before daughter replicon can be segregated. (Bate. D. A. and Maxwell A., 2007)

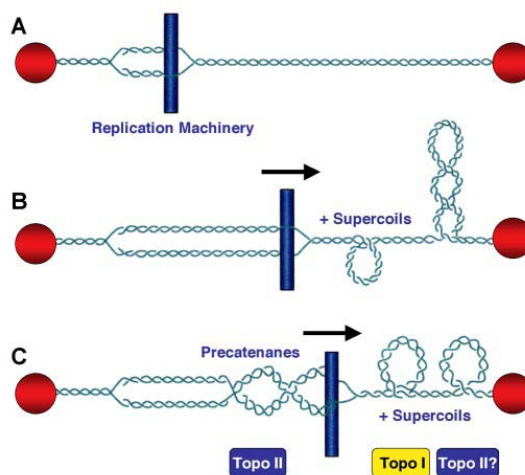


Figure 1. Model for topoisomerase function and topological stress associated with DNA replication. The replication machinery is represented by a rod moving through the double helix. DNA ends are anchored to hypothetical immobile structures existing in the nucleus. (A) upon initiation of DNA replication, the two strands of duplex DNA are separated, and the replication fork is formed. (B) movement of the replication machinery through the immobilized DNA template strands induces acute overwinding (*i.e.* positive supercoiling) ahead of the fork. (C) if the replisome rotates around the helical axis of the DNA, compensatory underwinding (*i.e.* negative supercoiling) behind the replication machinery allows some of the torsional stress in the prereplicated DNA to be translated to the newly replicated daughter molecules in the form of precatenanes. If these precatenanes are not resolved, they ultimately lead to the formation of catenated duplex daughter chromosomes. Topoisomerase

II is proposed to work primarily behind the fork to remove precatenanes. In contrast to replication models, drug models place topoisomerase II ahead of DNA tracking systems. (from McClendon K. A. *et.al.*, 2005)

2. DNA Topoisomerase

DNA topoisomerases are ubiquitous enzymes that affect many vital cellular processes by controlling the level of DNA supercoiling and removing the tangling of DNA strands via the concerted cleavage and religation of DNA strands. DNA topoisomerases utilize the hydroxyl group of an active site tyrosine side chain in the nucleophilic attack of the DNA phosphodiester backbone to form the covalent intermediate with the cleaved DNA. (McClendon K. A. *et.al.*, 2005)

2.1 Type of topoisomerase

2.1.1 Type I DNA topoisomerase

Type I topoisomerases act by generating a transient single – strand break in double helix, followed by either a single – stranded DNA passage event or controlled rotation about the break. As a result, these enzymes are able to relieve torsional stress (i.e remove superhelical twist) in duplex DNA. Type I DNA topoisomerases entail in all DNA processes that involve tracking systems and play important role in maintaining genomic integrity (McClendon K. A., *et. al.*, 2005).

Type I topoisomerases are subdivided into two subclasses: type IA topoisomerases which share many structural and mechanistic features with the type II topoisomerases and type IB topoisomerases which utilize a controlled rotary mechanism. Examples of type IA topoisomerases include topoisomerase I and topoisomerase III. Historically, type IB topoisomerases were referred to as eukaryotic topoisomerase I. The catalytic mechanism of Topoisomerase I involves a reversible transesterification reaction, as shown in Figure 2. In brief, following DNA binding the Topoisomerase I catalytic cycle is initiated by the nucleophilic attack of the phenolic hydroxyl group of the active site tyrosine (Tyr723 for human Topoisomerase I) on the scissile phosphate, which generates a covalent complex between the protein and the 3'-phosphate of the DNA via a phosphotyrosyl bond. This transient DNA single-strand break (i.e. cleavage complex) subsequently allows controlled rotation of the cleaved DNA strand around its intact complement, effectively removing any local helical tension. Once the DNA is relaxed, the covalent intermediate is reversed via a second transesterification reaction, wherein the free DNA 5'-hydroxyl acts as a nucleophile to attack the 3'-phosphotyrosyl linkage; thus restoring the continuity of the original DNA duplex. (Dexheimer S. T. *et al.*, 2008)

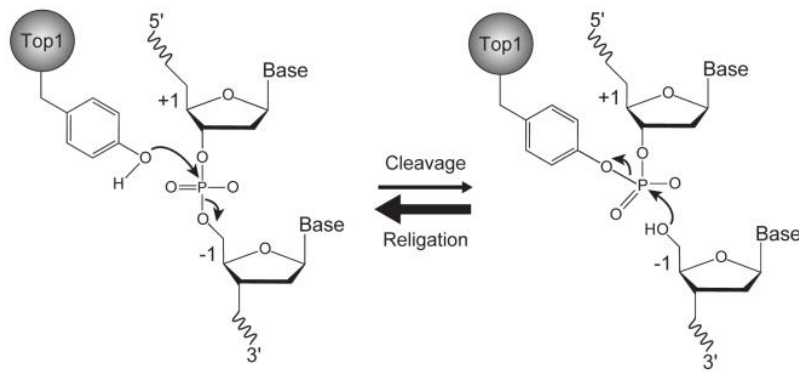


Figure 2 Human Topoisomerase I -mediated DNA cleavage and religation mechanisms. Tyr723 is the active site tyrosine involved in the transesterification reaction. The bases flanking the Topoisomerase I cleavage site are referred to as -1 and +1 for the bases at the 3' and 5' DNA termini, respectively (from Dexheimer S. T. *et al.*, 2008))

2.1.2 Type II DNA topoisomerase

Type II topoisomerase are ubiquitous enzymes that catalyze DNA topological changes by transporting one double-stranded DNA segment through another. They play essential roles in many aspects of DNA transaction *in vivo*, including chromosome condensation and segregation, and the removal of the supercoils generated during replication and transcription. In addition to such essential function in the cell, topoisomerase II is an important target of many widely used antibiotic and anti-tumor drugs. (Hu T. *et al.*, 1998.)

In mammals, there are two isozymes of DNA topoisomerase II: topoisomerase II α and topoisomerase II β . Both isozymes differ in their molecular mass (170 kDa versus 180 kDa respectively) and are encoded by separate genes. Topoisomerase II α and topoisomerase II β share about 70% in sequence homology. This is mainly in the N-terminal three-quarters of the protein sequence where the catalytic center is located. Unlike the N-terminal domain, the C-terminal quarter of protein containing highly charged residues shows much more sequence diversity. However, the C-terminal domain has shown to be vital for cell viability and is thought to be linked to regulation. (Meczes L. M. *et al.*, 2008)

Topoisomerase II α is an essential enzyme that plays important roles in DNA replication and chromosome segregation (Bender P. R. and Osheroff N., 2007). Several lines of evidence suggest that topoisomerase II α is the main isoform involved in mitotic processes. First, there is a positive correlation between the cellular concentration of topoisomerase II α and the rate of cell proliferation. Second, the expression of topoisomerase II α mRNA is higher in tissues containing proliferating cells. Third, the level of topoisomerase II α protein peaks at G2/M phase during the cell cycle and, finally, topoisomerase II α localizes to the centromeres and axes of metaphase chromosomes (Sakaguchi A. and Kikuchi A., 2003). Thus, topoisomerase II α is believed to be the isoform that functions in growth – dependent process, such as DNA replication and chromosome segregation. In contrast, the expression of β - isoform is independent of proliferation status and the enzyme dissociates from chromosome during mitosis. Topoisomerase II β cannot compensate for

the loss of topoisomerase II α in mammalian and appears to be dispensable at the cellular level since it is required for proper neural development in mice (McClendon K. A. and Osheroff N., 2007)

A major regulatory feature found in the C - terminal domain of topoisomerase II is nuclear localizations (NLS). Without these signals the enzyme is not able to localize to the nucleus, where it is essential during DNA replication, and cell viability is thus diminished or lost. Considering both isoforms of human Topoisomerase II, a strong NLS is found at 1454 – 1497 while another moderate NLS is also found at 1259 – 1296 of topoisomerase II α . In topoisomerase II β , nuclear localization signals are found in the C-terminal domain, with two strong NLSs at 1522-1548 and 1538 – 1573, and weaker sequence at 1294 – 1332. (Meczes L. M., et al., 2008)

2.2 Topoisomerase II domain structure

The primary structures of topoisomerase II α and II β are very similar and can be divided into three domains based on sequence homology with the bacterial type II enzyme, DNA gyrase (Figure 3.). The N-terminal domain (first ~670 amino acids) of topoisomerase II is homologous to the B-subunit of DNA gyrase (GyrB). This portion of the enzyme contains the site of ATP binding and hydrolysis. Crystal structures of this domain recently were solved for yeast topoisomerase II (Figure. 3) and human topoisomerase II α . (McClendon K. A. and Osheroff N., 2007)

The central domain (amino acids ~671–1200) of topoisomerase II is homologous to the A-subunit of DNA gyrase (GyrA). This portion of the enzyme contains the active site tyrosine (amino acid 805 for human topoisomerase II α and 821 for topoisomerase II β) required for DNA cleavage and ligation. A crystal structure for this domain in the absence of a DNA substrate was solved for yeast topoisomerase II (Figure 3). (McClendon K. A. and Osheroff N., 2007)

The C-terminal domain (amino acids ~1201–1521 for topoisomerase II α and ~1201–1621 for topoisomerase II β) is highly variable among species and between the two human isoforms. While it is dispensable for catalytic activity *in vitro*, this domain contains nuclear localization sequences and sites of phosphorylation. For many years, the C-terminal domain was thought to contribute little to the enzymatic activity of any type II topoisomerase. However, several recent studies suggest that this portion of the protein plays an intriguing and important role in the recognition of DNA geometry. As such, it may impart unique attributes, such as the ability to supercoil DNA or act in front of replication forks. Unfortunately, no structural information is available for the C-terminal domain of any eukaryotic type II enzyme at the present time. (McClendon K. A. and Osheroff N., 2007)

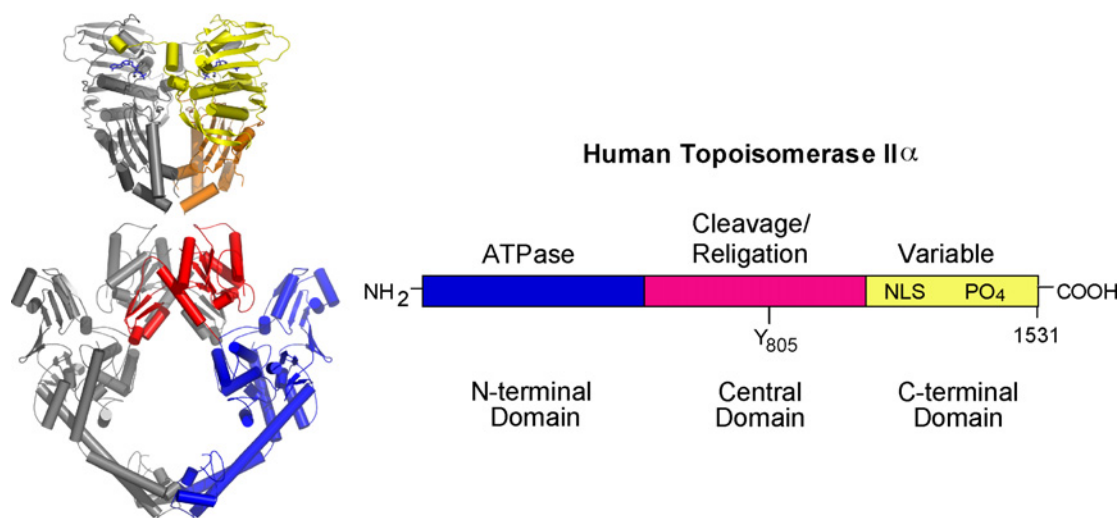


Figure 3. Structure of topoisomerase II. A ribbon diagram representing the crystal structure of a homodimer of yeast topoisomerase II is shown at left. The N-terminal domain is on the top (yellow and orange) and the central domain is on the bottom red and blue. At the present time, there is no structural information available for the C-terminal domain of any eukaryotic type II topoisomerase. The domain structure of human topoisomerase II α is shown at right. The N-terminal domain is homologous to the B-subunit of DNA gyrase (GyrB) and contains the site of ATP binding and hydrolysis. The central domain is homologous to the A-subunit of DNA gyrase (GyrA) and contains the active site tyrosine (Y805) required for DNA cleavage and ligation. The C-terminal domain is highly variable among species and contains nuclear localization sequences (NLS) and sites of phosphorylation (PO⁴). Although the C-terminal domain was thought to contribute little to the enzymological activity of any type II topoisomerase, several recent studies suggest that this portion of the protein plays an important role in the recognition of DNA geometry. (McClendon K. A. and Osheroff N., 2007)

2.3 Topoisomerase II catalytic cycle

Type II topoisomerase break both strands of DNA helix and pass a second double helix through the break (gate) in reaction that is dependent on the binding and hydrolysis of ATP. If the strand-passage reaction on circular DNA is intramolecular, the result is a change in the linking number of ± 2 (or, potentially, the introduction or removal of a knot) but if intermolecular, the result is the formation or resolution of catenanes. (Bates D. A. *et al.*, 2007)

The enzymes bind a DNA segment (the gate or G segment), which is cleaved in both strands with covalent attachment of the phosphate backbone to a pair of tyrosines on the enzyme, forming a transient DNA gate. The N-terminal domains of the enzyme complex (of the GyrB subunits in the case of gyrase) form a protein clamp that dimerizes in the presence of ATP, trapping another DNA segment (the transported or T segment), which is passed through the DNA gate. When the two segments (G and T) are part of different DNA molecules, this mechanism results in catenation/decatenation. When the two segments are part of the same molecule, strand passage generally results in DNA relaxation. (Wei H., *et al.*, 2005)

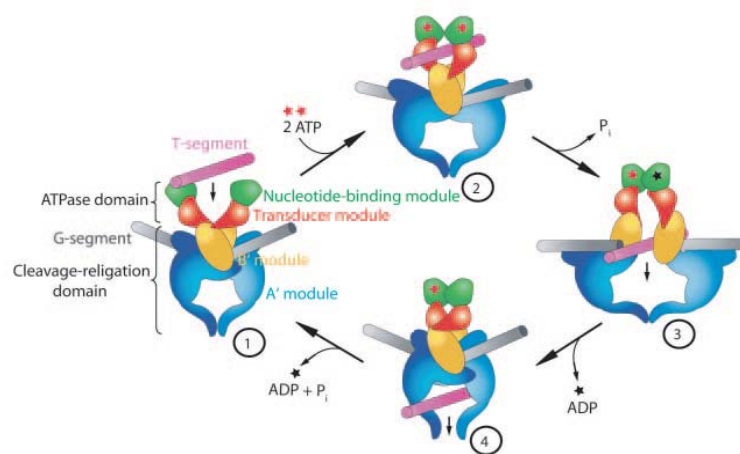


Figure 4. Model for nucleotide-dependent DNA translocation by Topoisomerase II. *Step 1*, G-segment DNA (gray) binds to the topoisomerase II dimer at the second gate of the cleavage-reunion core. *Step 2*, an incoming T-segment of DNA (pink) is captured in the upper cavity between the upper and middle gates by ATP (red star) binding-promoted dimerization. *Step 3*, hydrolysis and release of one of the two bound ATP molecules to ADP (black star) and P_i lead to an asymmetric retraction of the catalytic Lys-378, opening of the enzyme-bridged middle gate of the protein-DNA complex, and movement of the T-segment from the upper cavity formed in the ATPase domain into the lower cavity formed by the cleavage-reunion core. *Step 4*, hydrolysis of the remaining ATP and opening of the bottom gate allow for T-segment egress from the complex and resetting the system. (from Wei H., *et al.*, 2005)

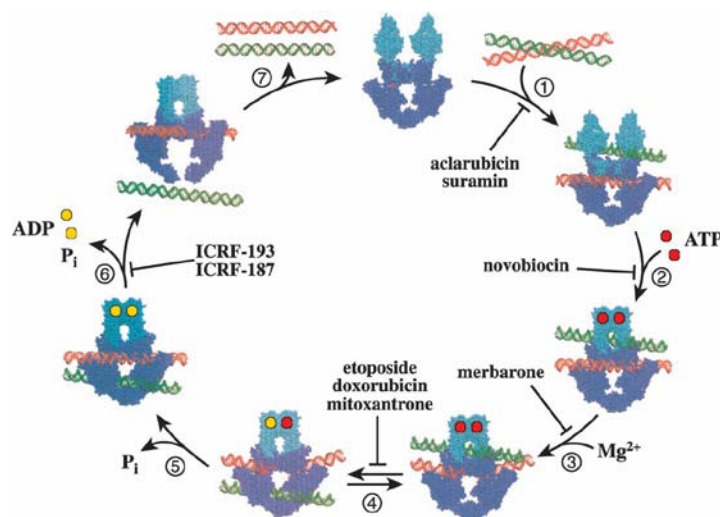


Figure 5. The catalytic cycle of DNA topoisomerase II. The ATPase domains of topoisomerase II are shown in light blue, the core domain in dark blue, and the active site tyrosine residue in red. The catalytic cycle is initiated by enzyme binding to two double-stranded DNA segments called the G segment (in red) and the T segment (in green) (Step 1). Next, two ATP molecules are bound, which is associated with dimerization of the ATPase domains (Step 2). The G segment is cleaved (Step 3) and the T segment is transported through the break in the G segment, which is accompanied by the hydrolysis of one ATP molecule (Step 4). The G segment is then religated and the remaining ATP molecule is hydrolyzed (Step 5). Upon dissociation of the two ADP molecules, the T segment is transported through the opening in the C-terminal part of the enzyme (Step 6) followed by closing of this gate. Finally, the N-terminal ATPase domains reopen, allowing the enzyme to dissociate from DNA (Step 7).

3. Classification of topoisomerase targeting anticancer drugs

Topoisomerase-targeting anticancer drugs can be divided into two broad classes that vary widely in their mechanisms of action. (Topcu Z., 2001). All topoisomerase II-directed agents are able to interfere with at least one step of the catalytic cycle (for details, see Figure 5.)

3.1 Class I topoisomerase inhibitors

These drugs act by stabilizing covalent topoisomerase–DNA complexes that are the intermediates during the catalytic cycle of the enzyme. Class I drugs are also referred to as “topoisomerase poisons” because they transform the enzyme into a potent cellular toxin. (Topcu Z., 2001). Topoisomerase II poisons increase the levels of enzyme–DNA cleavage complexes by two mechanisms. The first increases levels of enzyme–DNA cleavage complexes by interacting with topoisomerase II at the protein–DNA interface in a non-covalent manner, while the second acts by covalently modifying the enzyme. Some poisons act by inhibiting the ability of topoisomerase II to ligate the cleaved substrate. These agents not only increase the level of cleavage complexes, but also increase the lifetime of these complexes. Other poisons have little effect on the rate of enzyme mediated ligation and are believed to act primarily by enhancing the forward rate of cleavage complex formation. The exact mechanism by which this second group of drugs increases levels of DNA cleavage is unknown. They may specifically act to enhance the forward rate of DNA scission. Alternatively, they may have some effects on the DNA binding/dissociation equilibrium, as the level of topoisomerase II-mediated DNA cleavage is proportional to the amount of enzyme bound. (McClendon K. A. and Osheroff N., 2007)

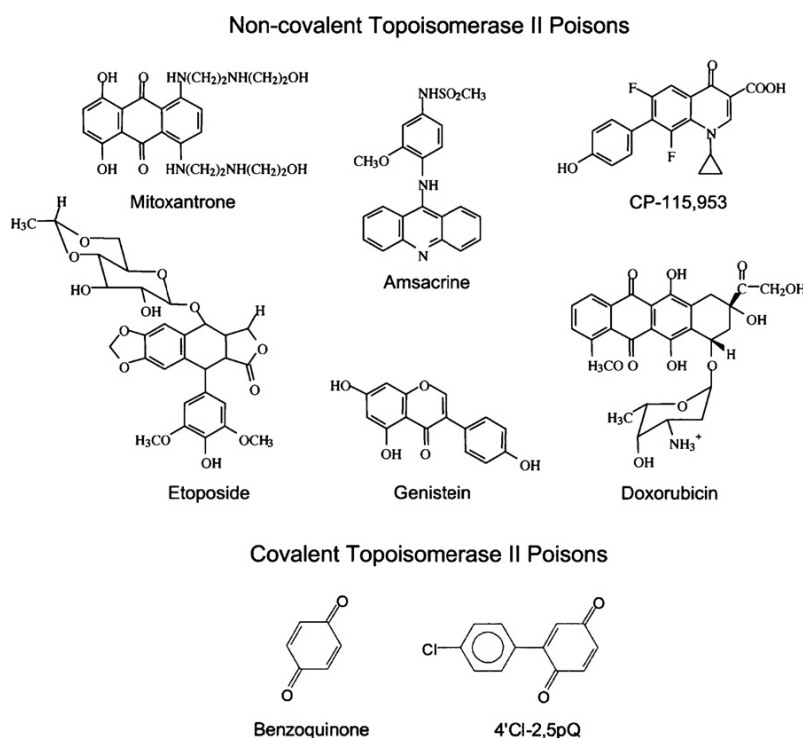


Figure 6. Structures of selected topoisomerase II poisons. Agents that act in a non-covalent fashion at the topoisomerase II–DNA interface are shown at the top. Quinones that act by covalently adducting the type II enzyme are shown at the bottom (from McClendon K. A. and Osheroff N., 2007)

3.2 Class II topoisomerase inhibitors

These drugs interfere with the catalytic function of the enzyme without trapping the covalent complex. The drugs in this class are referred to as “**topoisomerase inhibitor**” Catalytic topoisomerase II inhibitors are a heterogeneous group of compounds (for chemical structures, see Figure 7) that might interfere with the binding between DNA and topoisomerase II (aclarubicin and suramin), stabilize noncovalent DNA topoisomerase II complexes (merbarone, ICRF-187, and structurally related bisdioxopiperazine derivatives), or inhibit ATP binding (novobiocin). (Topcu Z., 2001).

4. Rhinacanthins

Rhinacanthins are naphthoquinone ester derivative isolated from the methanolic extract of the roots of the medicinal plant *Rhinacanthus nasutus* (Acanthaceae). In Thailand the roots and leave of this plant are used for the treatment of cancer. It has been reported that the synthesis of rhinacanthin-M, -N, and -Q showed significant cytotoxicities against KB, HeLa and HepG₂ cell line. Moreover, they showed some inhibitions toward enzyme Topoisomerase II (Kongkathip, N *et.al*, 2004). The inhibitory mechanism of these compounds towards Topoll is still unclear. According to Kongkathip , N et al 2004, these compounds were found at the DNA-binding domain of Topoll. Moreover, they also inhibited the relaxation activity of yeast Topoll. Therefore, acquiring knowledge on the mechanism of these compounds toward Topoll is still challenging.

In this report, eight compounds of naphthoquinone derivatives were used. Their structures are depicted in Figure 7.

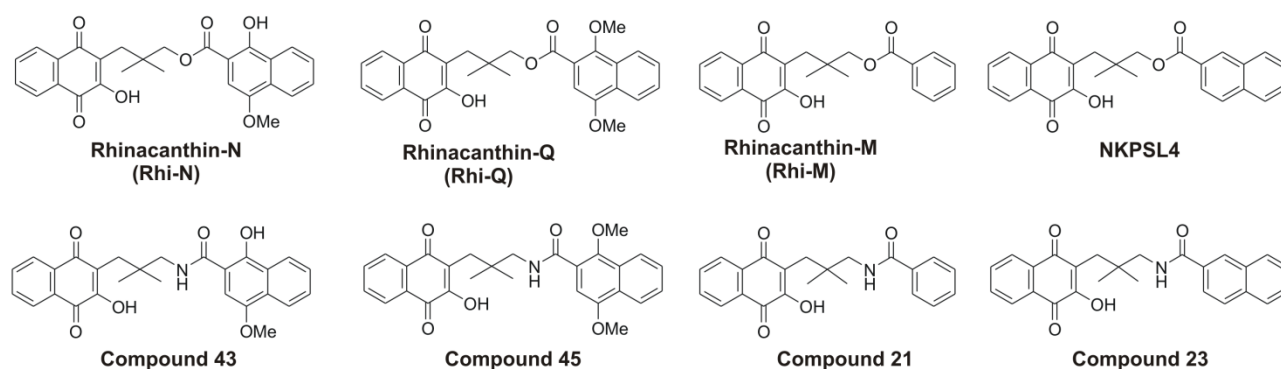


Figure 7. Chemical structures of naphthoquinone compounds used in this study.

5. Objectives

1. To understand the important of each domain of human Topoll α and Topoll β towards rhinacanthin such as naphthoquinone esters and amides
2. To identify the important amino acids involved in the specific interaction between each domain of human Topoll α and Topoll β and rhinacanthin compounds.

บทที่ 2 วิธีวิจัย

This chapter describes how to construct and produce recombinant proteins human Topoll α and Topoll β ATPase domain as well as investigate and determine the inhibition of rhinacanthins toward human Topoll and the kinetic parameters of hTopoll α -ATPase domain

1. Amplification of DNA fragments by polymerase chain reaction.

The plasmids pCM1 and YEPTOP2 (a gift from Prof. Osheroff, N.) were used as templates for the amplification of *ATPasehTopoll α* and *ATPasehTopoll β* by PCR, respectively. The PCR was performed by using *TaKaRa Ex Taq* DNA polymerase. Reaction mixtures containing 0.25 mM dNTP, 0.2 μ M 5' primer, 0.2 μ M 3' primer, 100 ng of Template DNA template, 5 μ l 10 x buffers, 1 unit of *TaKaRa Ex Taq* DNA polymerase and milliQ water to make total volume of 20 μ l. Table 1 shows the PCR condition to generate both *ATPasehTopoll α* and *ATPasehTopoll β* fragments while Table 2 displays primer sequences used for the PCR. The forward primers contain *Nhe*I restriction site and the reverse primers contain *Xho*I restriction site. These specific sites were used to clone the specific fragments into pET28b.

Table 1 PCR condition

Segment	Temperature	Time	Cycle
Pre-denaturation	95 °C	5 min	1
Denaturation	95 °C	1 min	
Annealing	55 °C	40 sec	25
Extension	72°C	3.54 min	
Final extension	72°C	5 min	1

Table 2 PCR primers used for the amplication of *ATPasehtopolI α* and *ATPasehtopolI β* . The yellow highlight represents the cutting site *Nhe*I, and the red highlight represents the *Xho*I cutting site.

Set of primer	Sequence	PCR product
F_hTopollA_29	5' GAGCAGCTAGCTCTGTTGAAAGAATCTATCAAAAG 3'	1.2 kb
R_hTopollA_428	5' GTCGGCCTCGAGTTATGAACACTTCTTGTTTAACTG 3'	
F_hTopollB_45	5' GAGCAGCTAGCTCTGTTGAGAGAGTGTATCAG 3'	1.2 kb
R_hTopollB_444	5' GTCGGCCTCGAGTTATGAACACTTCTTATTC 3'	

2. Ligation of *ATPasehTopoll α* and *ATPasehTopoll β* into pGEM-T Easy vector

The PCR products were purified by Nucleospin purification kit following the manufacturer instruction. The PCR fragments of *ATPasehTopoll α* and *ATPasehTopoll β* were ligated to pGEM-T Easy vector. The ligation reaction containing vector: insert molar ratio of 1:3, 1 U of T4 DNA ligase and added milliQ water to final volume of 10 μ l. The vector amount was approximately 50 ng per reaction. The ligation reaction was incubated at 4 °C overnight. The ligation reaction was transformed into *E.coli* (JM109) in LB plate containing appropriate concentration of ampicilin, IPTG, X-GAL. After transformation, the several white colonies were picked and confirmed by PCR (described below) and DNA sequencing.

3. Confirmation of plasmid pGEM-T-Easy-ATPasehTopoll α and pGEM-T-Easy-ATPasehTopoll β .

Colony PCR screening was employed to check the presence of the insert. The PCR was performed by using *Taq* DNA polymerase (Fermentus). The single colony of pGEM-T Easy-ATPasehTopoll α and pGEM-T Easy ATPasehTopoll β was used as a template. Reaction mixtures containing 0.2 mM dNTP, 0.2 μ M 5' primer, 0.2 μ M 3' primer, 0.2 mM MgCl₂, 2.5 μ l 10x buffer, 0.5 unit of *Taq* DNA polymerase and milliQ water to make up a total volume of 20 μ l. The PCR condition was shown in Table 1. The PCR screening products were checked by 0.8% agarose gel electrophoresis.

4. Construction of *ATPasehTopoll α* and *ATPasehTopoll β* into pET28b vector

The plasmids pGEM-T-Easy-ATPasehTopoll α and pGEM-T-Easy-ATPasehTopoll β digested with *Nhe*I and *Xho*I were ligated with corresponding size of pET28b which was previously digested with *Nhe*I and *Xho*I. The constructed plasmid was called pET28b-ATPasehTopoll α and pET28b-ATPasehTopoll β . After the ligation, the recombinant plasmids were transformed into *E. coli* (JM109) and selected on LB plate containing the appropriate concentration of kanamycin. After transformation, the plasmids were extracted by GeneJET plasmid miniprep kit and the insertion of the interested fragment was confirmed by PCR screening.

5. Expression of ATPase_hTopoll α and ATPase_hTopoll β

The plasmids pET28b-ATPase-hTopoll α and pET28b-ATPase-hTopoll β were transformed into *E.coli* BL21 (DE3). Transformant colonies were selected in LB agar containing 50 μ g/ml kanamycin. Then transformant colony was picked and inoculated in 2.5 ml LB medium containing 50 μ g/ml kanamycin as a starter cell. The starter cell culture was diluted into 250 ml LB medium supplement 50 μ g/ml kanamycin. The cell culture was grown in shaking incubator at 37°C until OD₆₀₀ reached 0.4-0.6. The expression of ATPase-hTopoll α protein was induced by adding IPTG at a final concentration of 1 mM. The cell culture was then incubated at 30°C for overnight. For the time course experiment, an aliquot of the induced culture containing approximately 10⁸ cells (1 ml of 1 OD₆₀₀) was collected at 0,1,2,3 and 16 hours. The cells were harvested by centrifugation at 3,000 rpm. The expressed protein was analyzed by 12% SDS-PAGE.

6. Purification of ATPase-hTopoII α

After the expression of ATPase-hTopoII α , cells were harvested by centrifugation at 3,000 rpm at 4°C. Cell pellets were resuspended in lysis buffer (50 mM Tris-HCl pH 7.5, 0.5 M NaCl, 0.5 mM Imidazole). Lysis buffer 3 ml was used for 100ml of cell culture. The cells were subsequently sonicated using pulse amplitude (on 10 sec, off 5 sec) for 30 min. After the sonication, the cell lysate was centrifuged at 15,000 rpm for 30 min at 4°C. The supernatants were applied to 1 ml HiTrap Chelating Ni²⁺ column which was equilibrated with washing buffer (50 mM Tris-HCl pH 8, 0.5 M NaCl, 0.03 M Imidazole). The column was washed with washing buffer to remove non-specifically bound protein. The 6xHis ATPase-hTopoII α protein was eluted by an increasing imidazole gradient of elution buffer (50 mM Tris-HCl pH8, 0.5 M NaCl, 0.5 M Imidazole). Protein fractions were analyzed by 12% SDS-PAGE. Protein fractions containing 6xHis ATPase-hTopoII α protein were pooled and dialyzed in 20 mM Tris-HCl pH 8 and 50 mM NaCl to remove imidazole.

7. Estimation of protein concentration

Concentration of protein was measured in conjunction with Beer-lambert law, $A = \epsilon bc$ where A , ϵ , b and c were absorbance, molar extinction coefficient, path length and molar concentration respectively. Extinction coefficient of ATPase-hTopoII α ϵ_{280} was $54,235 \text{ M}^{-1} \text{ cm}^{-1}$.

8. Binding study of ATPase-hTopoII α

Fluorescence titration method was performed by making microlitre addition of the ligands such as 1 mM rhinacanthin derivatives to 400 μl of 2.5 μM ATPase-hTopoII α in 20 mM Tris-HCl pH8, 50 mM NaCl. An excitation wavelength of 295 was used and the fluorescence emission spectra were recorded between 315-400 nm.

The apparent K_d values for the ligand binding were obtained by fitting the following equation:

$$\% \Delta F_{\text{obs}} = \frac{\Delta F_{\text{max}} [L]_o}{K_d + [L]_o}$$

where $\% \Delta F_{\text{obs}}$ is the change in fluorescence intensity, ΔF_{max} is the maximum attainable change in fluorescence intensity, $[L]_o$ is the total molar concentration of the ligand, and K_d is the dissociation constant.

9. Topoisomerase assay

Human Topoisomerase II α activity was determined using Topoisomerase II assay kit (TopGEN Inc.). The reaction mixture containing 60 ng of kintoplast DNA (kDNA) and 2 units of hTopoII α was

incubated with and without naphthoquinone derivatives at 37 °C for 1 h in complete assay buffer (50 mM Tris-Cl pH 8.0, 150 mM NaCl, 10 mM MgCl₂, 0.5 mM dithiothreitol, and various concentrations of ATP). Doxorubicin was used as a positive control while decatenated and linearized kDNA were used as markers. The reaction in a final volume of 20 µl was stopped by adding stop buffer/gel loading dye (1% Sarkosyl, 0.025% bromophenol blue and 5% glycerol). Reaction products were run on 1% agarose gel in 0.5x TBE buffer with 0.5 µg/ml ethidium bromide included in the gel. Electrophoresis was performed at 50 V for 1.30 h. After electrophoresis, the gel was destained with distilled water for 30 min and photographed over a UV transilluminator using DNR Bio-Imaging system. One unit of Topoisomerase II is defined as the amount of enzyme that decatenates 0.2 µg of kDNA in 30 min at 37 °C.

10. ATPase assay

To ascertain the ATPase activity, ATPase measurement was carried out by the PK/LDH couple assay described previously with some modifications [28]. The reaction mixture of 200 µl contained 10 mM Tris-Cl pH 7.5, 50 mM NaCl, 50 mM KCl, 5 MgCl₂, 0.1 mM NADH, 2 mM phosphoenolpyruvate, 3 units of pyruvate kinase, 4 units of lactate dehydrogenase and various concentrations of ATP. Both ATP and the reaction mixture were pre-equilibrated at 30 °C for 5 min before the measurement. Finally, 0.1 µM of the enzyme was added into the reaction mixture. The decrease in NADH, which was directly proportional to the rate of ATP hydrolysis, was monitored by measuring the absorbance at 340 nm in a microplate reader spectrophotometer (Sunrise-basic TECAN). In order to determine the inhibition of ATPase activity by the naphthoquinone compounds, 50 µM of the compounds were added to each reaction mixture and pre-equilibrated at 30 °C for 5 min before the measurement. All assays were performed in triplicate.

11. Molecular docking analysis

To better understand how the ligands bind to the enzyme, molecular docking analysis was performed. The crystal structure of ATPase domain of human TopoII α was obtained from the protein data bank (pdb code 1ZXM) [29]. The structures of naphthoquinone compounds were built using Sybyl 7.3 program. Docking analysis was performed with Autodock 4.0 program. The grid was chosen to be sufficiently large to include the ATP binding site as a whole with spacing of 0.375 Å and a grid size of 60 x 60 x 60 points along the x, y and z axes. The center of the ligands was positioned at the grid center. The search parameter used was Lamarckian Genetic Algorithm (LGA) with 100 runs. The population size was set at 150 and the number of energy evaluations was 2.5 million. Three dimensional structures and molecular surfaces of hTopoII α -ATPase-naphthoquinone compounds with the best-docked conformation were visualized and analyzed by PyMOL.

บทที่ 3 ผลการวิจัยและวิจารณ์

1. Amplification of DNA fragments by polymerase chain reaction.

Gene coding for ATPase domain of human TopoII α and TopoII β was amplified by PCR using the PCR condition and primers as shown in Table 1 and 2. Figure 8 shows the PCR products of *ATPaseTopoII α* and *ATPaseTopoII β* fragments. The sized of the products were around 1.2 kb as expected for *ATPaseTopoII α* and *ATPaseTopoII β* . As can be seen, the PCR products were relatively cleaned so the PCR products were then cleaned by PCR purification kit prior to ligate to the pGEM-T-Easy vector.

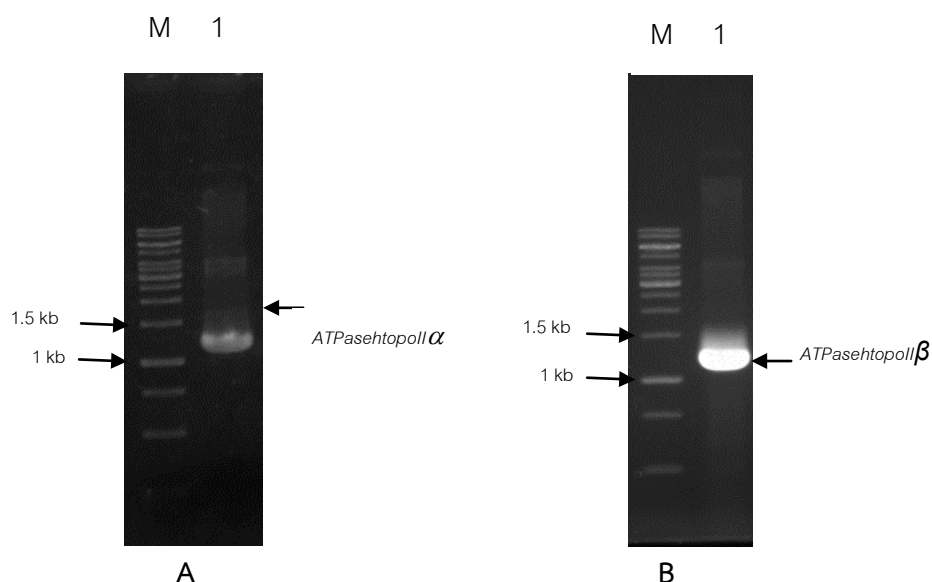


Figure 8 PCR product for *ATPaseTopoII α* and *ATPaseTopoII β* . (A) PCR product for *ATPaseTopoII α* . M, GeneRuler Express DNA Ladder (Fermentus); Lane 1, *ATPaseTopoII α* . (B) PCR product for *ATPaseTopoII β* . M, GeneRuler Express DNA Ladder (Fermentus); Lane 1, *ATPaseTopoII β* .

2. Ligation of *ATPaseTopoII α* and *ATPaseTopoII β* fragment into pGEM-T Easy vector

The cleaned PCR products were used to ligate with pGEM-T-Easy as described in the method section. The transformant from the ligation reaction was picked and screened. The PCR screening products from the selected transformant were checked by 0.8% agarose gel electrophoresis as shown in Figure 9. The size of the recombinant plasmids containing *ATPaseTopoII α* and *ATPaseTopoII β* was expected to show the band of 1.2 kb (Figure 9). The result confirmed that the recombinant plasmids contained the insert gene. The plasmid pGEM-T Easy-*ATPaseTopoII α* was further confirmed by DNA sequencing and the clone 8 of pGEM-T Easy-*ATPaseTopoII α* contained no mutation in the *ATPaseTopoII α* region (data not shown). However, the sequencing result of pGEM-T-Easy-*ATPaseTopoII β* is being investigated. Therefore, only the clone 8 of pGEM-T Easy-*ATPaseTopoII α* will be used as a template to construct pET-28b-*ATPaseTopoII α* .

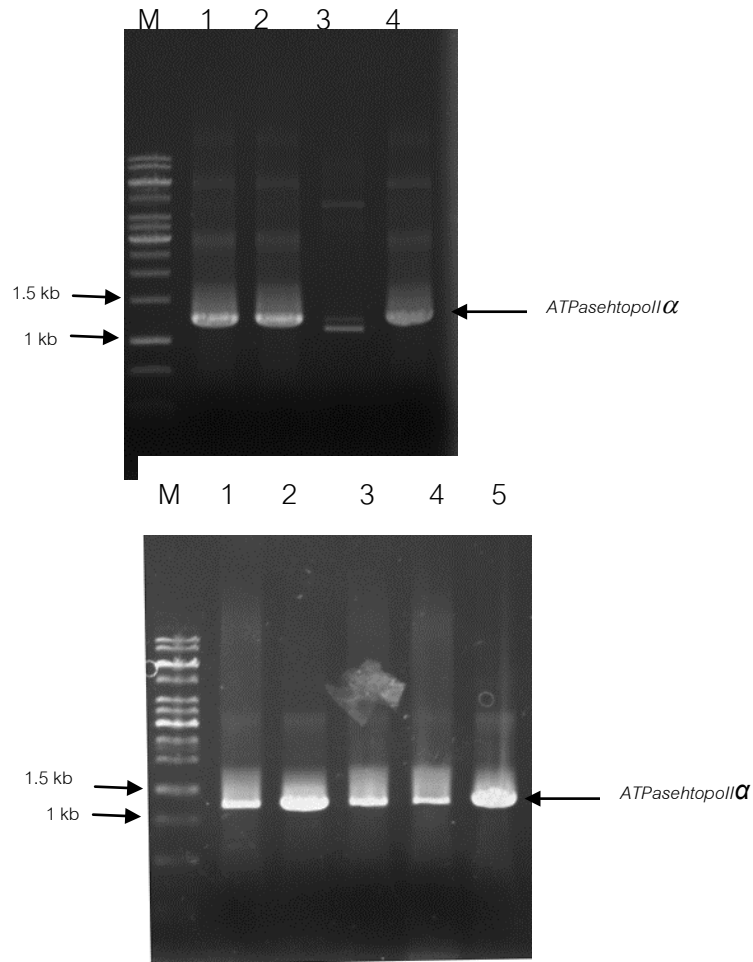


Figure 9 PCR screening of pGEM-T Easy-ATPaseTopoll α and pGEM-T Easy-ATPaseTopoll β . (A) PCR screening of pGEM-T Easy-ATPaseTopoll α . M, GeneRuler Express DNA Ladder (Fermentus); Lane 1-4 PCR screening of pGEM-T Easy-ATPaseTopoll α clone 5-8 respectively. (B) PCR screening of pGEM-T Easy-ATPaseTopoll β . M, GeneRuler Express DNA Ladder (Fermentus); Lane 1-5 PCR screening of pGEM-T Easy-ATPaseTopoll β clone 1-5 respectively.

3. Construction of *ATPasehtopolII α* into pET28b vector

After the sequence of *ATPhTopoll α* in pGEM-T Easy-ATPaseTopoll α was confirmed, the insert digested with *NheI* and *XhoI* was later ligated with the pET28b previously digested with the same restriction enzyme. The transformant from the ligation reaction was screened by PCR. Figure 10 shows the PCR product of *ATPasehtopolII α* in pET28b- *ATPasehtopolII α* clone 1-5. The result here indicated that all plasmids contained the correct insert. Therefore only clone 1 of pET28b- *ATPasehtopolII α* will be chosen to use in the protein expression experiment.

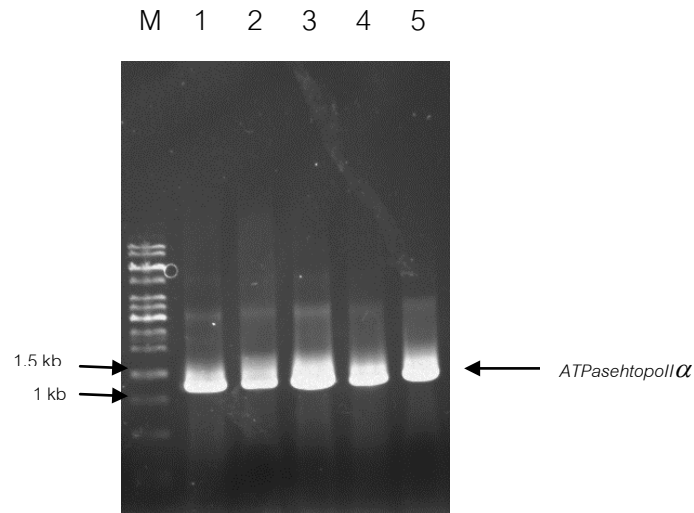


Figure 10 PCR screening of pET28b-ATPasehTopoll α . M, GeneRuler Express DNA Ladder (Fermentus); Lane 1-5 PCR screening of pET28b-ATPasehTopoll α . M, GeneRuler Express DNA Ladder (Fermentus); Lane 1-5 PCR screening of pET28b-ATPasehTopoll α clone 1-5 respectively.

4. Protien expression of ATPase-hTopoll α and ATPase-hTopoll β

To express ATPase-hTopoll α and ATPase-hTopoll β , *E.coli* BL21(DE3) was used as a suitable host for protein expression. Addition of IPTG in the growing culture induces T7 RNA polymerase production, which in turn transcribes the target DNA in the plasmid. In order to find a suitable time for the over expression of ATPase-hTopoll α and β , a time course experiment was carried out and the results are shown in Figures 11-12. After the single colony of *E.coli* BL21(DE3) containing ATPase-hTopoll α/β plasmid was picked and grown in LB broth containing 50 $\mu\text{g}/\text{ml}$ kanamycin at 37 $^{\circ}\text{C}$ until optical density at 600 nm reached 0.6, the cell culture was induced with 1 mM IPTG and grown at 30 $^{\circ}\text{C}$ for induced recombinant proteins. The *E.coli* cell lysate containing recombinant proteins was collected at 0, 1, 2, 3 and 16 hr after induction. As can be seen in Figure 8 and 9, the ATPase-hTopoll α/β proteins (~45 kDa) could be expressed at 30 $^{\circ}\text{C}$ and the maximal expression could be observed at 16 hr after induction.

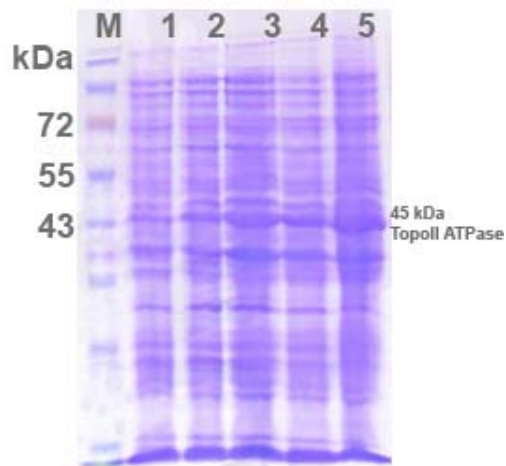


Figure 11 Time course expression analysis of ATPase_htopolI α protein induced with 1 mM IPTG at 30°C. 12% SDS-PAGE was employed. M, PageRuler prestained protein ladder (Fermentas); Lane1-5, Expression of ATPase_htopolI α protein for 0 hr, 1 hr, 2 hr, 3 hr and 16 hr respectively.

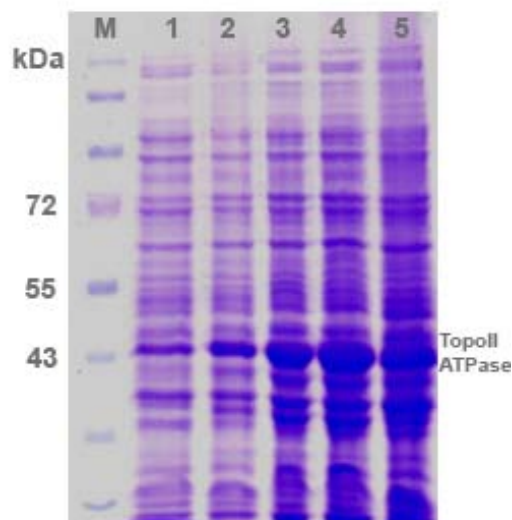


Figure 12 Time course expression analysis of ATPase_htopolI β protein induced with 1 mM IPTG at 30°C. 12% SDS-PAGE was employed. M, PageRuler prestained protein ladder (Fermentas); Lane1-5, Expression of ATPase_htopolI β protein for 0 hr, 1 hr, 2 hr, 3 hr and 16 hr respectively.

5. Protein purification

Since both proteins can be expressed in the small scale, the large scale protein preparation was carried out. Since the expressed both ATPase-htopoll proteins were fused with 6xHis tag at N-terminus, so the proteins could be purified using Ni^{2+} affinity column. However, after the cells were broken by sonication method, it was found that the ATPase-hTopoll β protein was expressed as insoluble inclusion bodies. Therefore, only the ATPase-hTopoll α protein can be further purified. SDS-PAGE analysis of purified ATPase-hTopoll α protein is shown in Figure 13. This result revealed that ATPase-hTopoll α protein was successfully purified and the molecular weight of the purified protein was about 45 kDa. The concentration of purified protein was measured using the extinction coefficient of $54,235 \text{ M}^{-1} \text{ cm}^{-1}$ for ATPase-hTopoll α .

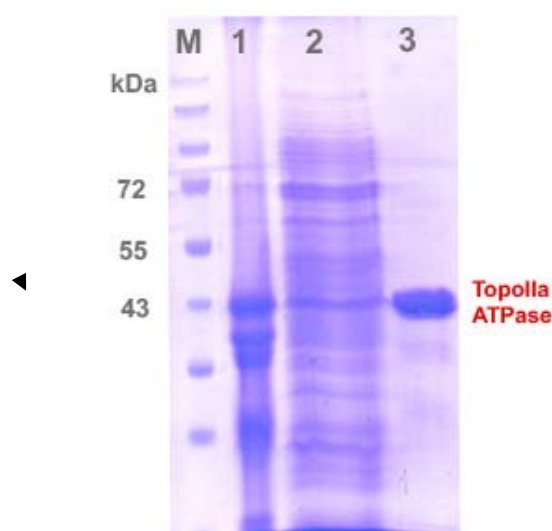


Figure 13 purification analysis of ATPase_htopoll α . M, PageRuler prestained protein ladder (Fermentas); Lane 1, insoluble; Lane 2, flow through sample from FPLC; Lane 3, purified ATPase_htopoll α .

6. Fluorescence binding study of ATPase-hTopoll α

Tryptophan fluorescence study was chosen to identify the binding affinity of novel naphthoquinone amides to ATPase-hTopoll α protein. The excitation wavelength at 295 nm was used to excite Trp residues and the emission spectrum between 315 - 400 nm was observed. The K_D value can be calculated using equation as described in methodology section (2.8). When naphthoquinone aromatic amides were added into the solution, a quenching in fluorescence intensity was observed as shown in Figure 14. The maximal emission for Trp was at 344 nm for ATPase-hTopoll α protein alone and all ligands were not fluorescent under the experimental condition. The addition of the compounds did not change the maximal emission peak of Trp. The K_D values could be determined by plotting the change of percent fluorescence intensity and concentration of rhinacanthins using equation in methodology section (2.8). Table 3 shows the K_D values of each naphthoquinone derivatives. According to the K_D values, it seems that these novel

rhinacanthins with the amide linkage moderately bind to ATPase-hTopoll α protein which relates to their cytotoxicity and the naphthoquinone esters seem to bind slightly stronger than the amides.

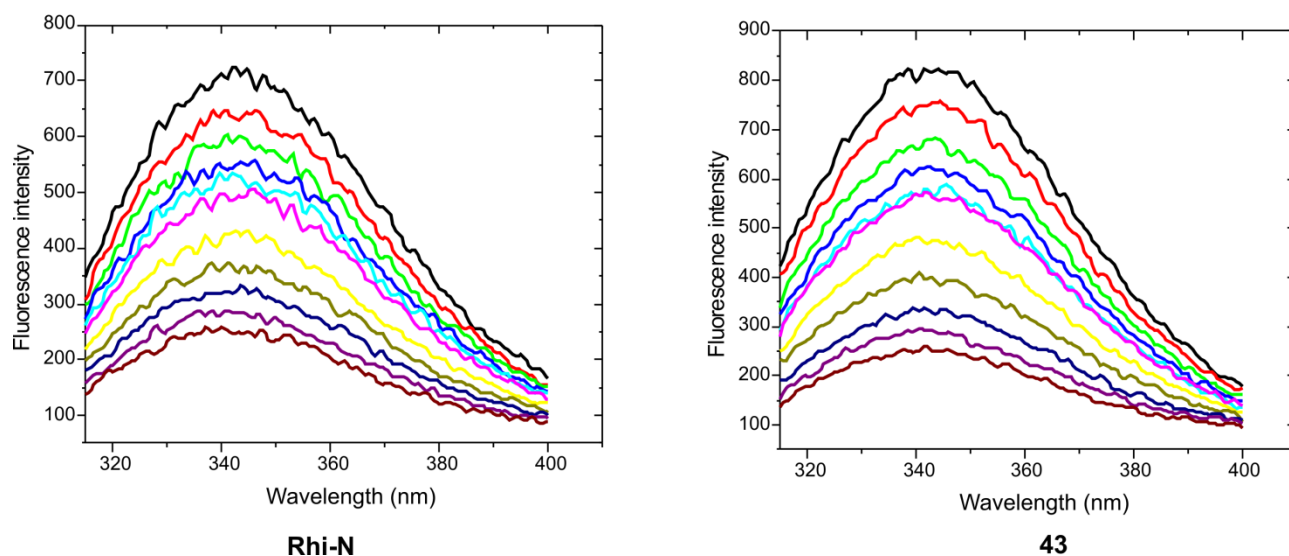


Figure 14 Fluorescence spectra of ATPase-hTopoll α upon the addition of Rhi-N and compound **43**.

Table 3. Dissociation constants (K_d s). The K_d values were obtained from the fluorescence binding studies in the presence of 5 mM ATP.

Naphthoquinone esters	K_d (μ M)	Naphthoquinone amides	K_d (μ M)
Rhi-N	23 ± 2	Compound 43	30 ± 3
Rhi-Q	22 ± 1	Compound 45	26 ± 4
Rhi-M	37 ± 4	Compound 21	50 ± 7
NKPSL4	23 ± 2	Compound 23	45 ± 4

7. Naphthoquinone affecting DNA relaxation in ATP concentration dependence.

Naphthoquinone ester derivatives have been reported to be able to inhibit the DNA relaxation activity of Topoll. Despite rhinacanthins being suggested to be able to interact with the DNA binding domain, it cannot be ruled out that they also interact with the ATPase domain. Expoxide ring-opened xanthone derivatives, for instance, have shown ATPase inhibitory activity inversely proportionally to ATP concentration (Jun KY et al 2011). Therefore, it would be of interest to determine if the inhibitory action of naphthoquinone compounds on DNA relaxation is also proportional to ATP concentration. In Topoisomerase II assay, compound **43** (a naphthoquinone amide analog to Rhi-N) was chosen. Recently, naphthoquinone aromatic amides also attract our interests due to their stability towards the hydrolysis. Therefore, a series of naphthoquinone aromatic amide were synthesized and their cytotoxicities against cancer cell lines and Vero cells

were tested [25]. The decatenation assay was used to determine the inhibitory activity of compound **43** and hTopoll α . The enzyme catalyzes the ATP-dependent decatenation of long-chained, catenated DNA molecules into free relaxed and supercoiled forms. As seen in Figure 15, compound **43** exhibited strong hTopoll α inhibitory activity in the presence of 2 mM ATP. However, when ATP concentrations increased, hTopoll α inhibitory activity of compound **43** reduced. It is likely that the tested compound compete with ATP. This result is in agreement with the fluorescent binding assay.

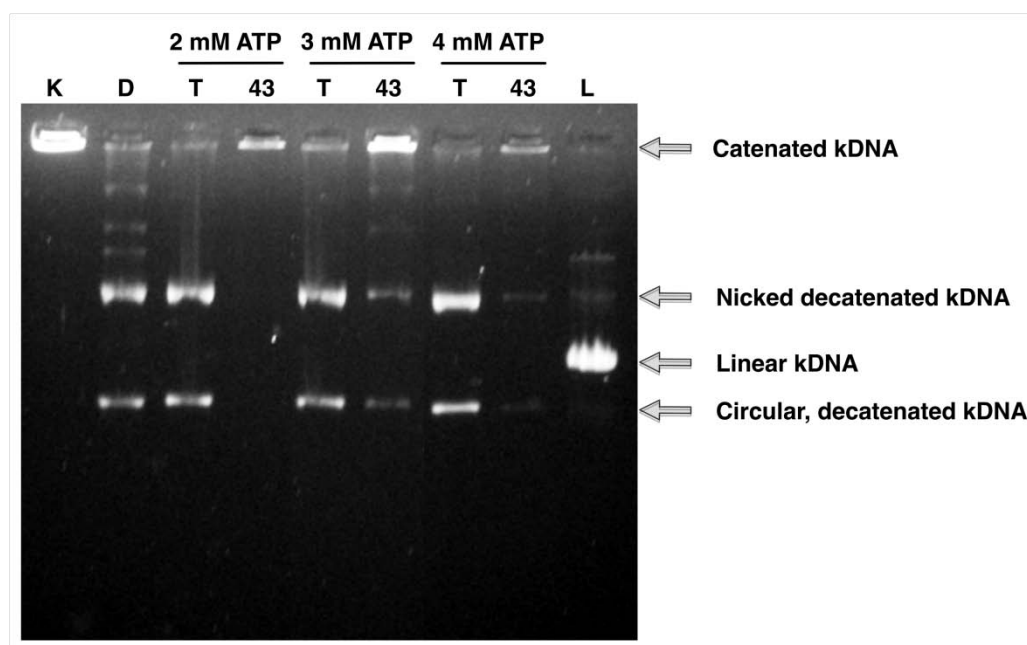


Figure 15 Topoisomerase II α inhibitory activity of naphthoquinone aliphatic amide **43**. The compound was examined in final concentration of 50 μ M. Lanes K: kDNA only, Lane D: decatenated kDNA only, Lane T: kDNA + hTopoll α , Lanes **43**: kDNA + hTopoll α + compounds **43**. The concentrations of ATP used in each reaction are indicated above each lane. Catenated kDNA does not migrate out of the loading wells.

8. Effect of naphthoquinones on hTopoll α -ATPase

Etoposide has been proposed to bind both to the N-terminal domain and the core domain of Topoll but does not eliminate ATP binding. STD-NMR experiment has also revealed that the binding of etoposide to human Topoll α is driven by the interactions with the A-ring and the B-ring and by stacking interactions with the E-ring of the etoposide. Etoposide can inhibit the rate of either the first ADP release or the hydrolysis of the second ATP. Unlike etoposide, bisdioxopiperazines such as ICRF-187 have been found to bridge and stabilize a transient dimer interface between two ATPase domains but does not compete with the ATP-binding. The multiple mutations both in the N-terminal domain and the core domain can cause the resistance to bisdioxopiperazines. Bisdioxopiperazine derivative such as ICRF-193 has not significantly inhibited

ATPase hydrolysis of the 52 kDa of human Topoll. Based on our fluorescence binding study, the inhibition activity of naphthoquinone compounds towards ATPase protein was next examined by ATPase assay in the absence of DNA. Figure 16 shows the ATPase activity of hTopoll α -ATPase protein in the absence and presence of naphthoquinone compounds. All naphthoquinone compounds can more or less interfere with ATPase activity of ATPase domain of hTopoll α . Additionally, the naphthoquinone aromatic esters seemed to inhibit ATPase activity slightly higher than the naphthoquinone aromatic amides. According to the result, the rate of enzyme-catalyzed ATP hydrolysis in the presence of the naphthoquinone compounds was slightly inhibited but not vastly different compared with that in the absence of the compounds.

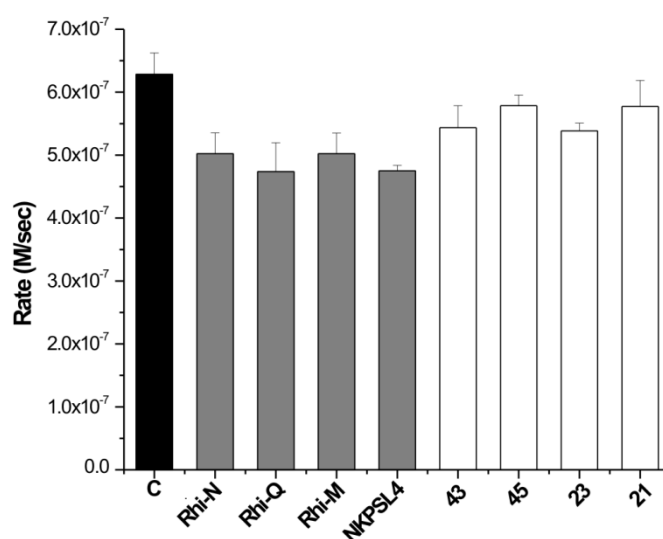


Figure 16 ATPase activity of hTopoll α -ATPase in the presence of naphthoquinone compounds. The ATPase activity was measured at 2 mM ATP and 50 μ M naphthoquinone compounds in the absence of DNA. The data shown are means \pm S.D. for three independent experiments.

9. Molecular docking of naphthoquinone derivatives and hTopoll α -ATPase

To visualize how the ligands position in hTopoll α -ATPase and to examine the binding mode of the naphthoquinone compounds, molecular docking studies were carried out for Rhi-N and compound **43** with ATP-binding domain of hTopoll α using the Autodock 4.0 program. The docking analysis showed that the compounds fit into the ATP-binding site. The position of the naphthoquinone ring in both compounds aligns with the phosphate backbone of ATP but only the naphthalene moiety of rhinacanthin-N overlaps well with the purine ring of ATP (Figure 17A and 17B).

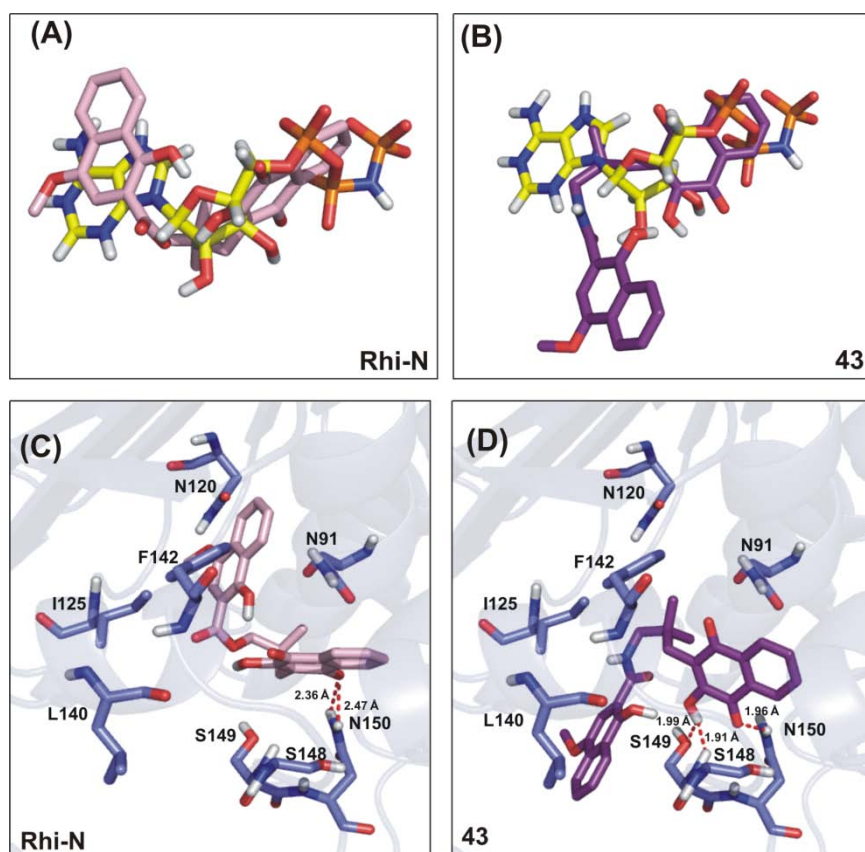


Figure 17. Overlay structure of AMP-PNP and Rhi-N (A) and compound **43** (B) in ATPase domain of hTopoll α . The molecules are shown in stick and colored by elements (carbon: light pink for Rhi-N, violet for compound **43** and yellow for AMP-PNP; oxygen: red; hydrogen: white, nitrogen: blue and phosphate: orange). (C) and (D) are view of Rhi-N and compound **43** in ATPase domain of hTopoll α , respectively. The amino acids involved in the interaction of rhinacanthins are depicted in stick representation and colored by elements (carbon: blue slate; oxygen: red; hydrogen: white and nitrogen: blue). The dotted red lines represent the hydrogen bonding interaction.

Figure 17C and 17D represents the arrangement of the naphthoquinone compounds in the binding pocket. Both Rhi-N and compound **43** are surrounded by hydrophobic amino acid residues such as I125, F142 and L140 for naphthalene moiety, and small polar uncharged amino acid residues such as S148, S149 and N150 for naphthoquinone ring. In Rhi-N-ATPase complex, the NH side chain of N150 forms a bifurcated-hydrogen bond with the C1-carbonyl of the naphthoquinone ring, whereas in the Compound **43**-ATPase complex, two types of hydrogen bonds are observed: a typical hydrogen bond between the NH side chain of N150 and C4-carbonyl of the naphthoquinone ring and a bifurcated hydrogen bond between C3-OH of the naphthoquinone ring and OH side chain S149 and main chain O of S148. It is important to note that compound **43** shows the existence of close contact with shorter hydrogen bonds to the naphthoquinone moiety than Rhi-N. These observed hydrogen bond networks have been seen in the interaction between xanthone analog and ATP-binding domain of hTopoll α [32]. Interestingly, the arrangement of Rhi-N in the ATP binding site is similar to that of xanthone analog. The hydrogen bond interaction with S149 is also observed in the purine scaffold derivative. According to the structure of hTopoll α -

ATPase –AMPPNP complex, the adenine ring is held through hydrogen bonding to a side chain carbonyl of N120 and S148 donates a hydrogen bond to the β -phosphate. However, the key catalytic general base, D87, is not observed as a part of the interaction between naphthoquinone compounds and the protein. Since the ATP hydrolysis assay showed neither naphthoquinone compounds dramatically inhibit the ATPase activity, other mechanisms in addition to binding to the ATPase domain may exist. The molecular docking here can provide an insight for a new design of the novel naphthoquinone compounds in order to improve the binding affinity of the compounds and to compete effectively with ATP.

บทที่ 4 สรุปและเสนอแนะ

This report presents the effect of naphthoquinone derivatives as potential Topoisomerase II inhibitors. Naphthoquinone aromatic esters and amides have been tested against cancer cell lines showing potent cytotoxicities. In this report, a selected compound **43** exhibited hTopoll α inhibition when 2 mM ATP was present. The ATP competition assay revealed that the inhibition of hTopoll α by compound **43** was inversely proportional to the ATP concentration. This has led to the further study on hTopoll-ATPase domain. Attempts were made for protein expression of both hTopoll α -ATPase and hTopoll β -ATPase but only hTopoll α -ATPase domain was obtained in sufficient amount. The inhibition of ATPase activity of hTopoll α -ATPase by naphthoquinone compounds was evaluated. The naphthoquinone aromatic esters exhibited slightly higher ATPase activity inhibition than the naphthoquinone aromatic amides. Since the rate of enzyme-catalyzed ATP hydrolysis in the presence of the naphthoquinone compounds was slightly inhibited compared with that in the absence of the compounds, other mechanisms in addition to binding to the ATPase domain may exist for the inhibition of hTopoll α activity. In order to identify where naphthoquinone compounds bind to the ATPase domain, molecular docking analysis of ATPase domain of hTopoll α was employed. The docking indicated that the compounds position in the same manner of AMP-PMP but only a few hydrogen bond interactions were detected. In addition to the positions of the naphthoquinone compound, the docking experiment revealed different spatial arrangement between naphthoquinone esters and amides as well as some important amino acid residues involved in the binding of the naphthoquinone compounds.

In summary, our results provide the first information about the interaction of the ATPase domain with naphthoquinone compounds. We have shown that the compounds can interfere with Topoll activity in the ATP dependent manner. Biological experiments confirmed that the compounds bind to hTopoll α -ATPase in the presence of ATP. Additionally, naphthoquinone esters can slightly reduce the ATPase activity further than naphthoquinone amides. Given the importance of hTopoll inhibitors as potential anti-cancer drugs, the further elucidation of the mechanism of action of the naphthoquinone esters and amides inhibitory activity described here and the development of further novel naphthoquinone derivatives which act as hTopoll α inhibitors is essential

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