มหาวิทยาลัยมหิดล
คณะวิทยาศาสตร์

60 YEARS MAHIDOL UNIVERSITY SCIENCE

งานประชุมวิชาการประจำปี
สมาคมเภสัชวิทยาแห่งประเทศไทย
ครั้งที่ 40

Abstracts and Proceedings

"นวัตกรรมทางเภสัชวิทยา"
(Innovation in Pharmacology)

26–28 เมษายน พ.ศ. 2561
คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล พญาไท

จัดโดย : ภาควิชาเภสัชวิทยา
คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล
สมาคมเภสัชวิทยาแห่งประเทศไทย

ABSTRACTS & PROCEEDINGS

การประชุมวิชาการประจำปีสมาคมเภสัชวิทยาแห่งประเทศไทยครั้งที่ 40

26-28 เมษายน 2561

Innovation in Pharmacology

จัดการประชุมโดย
สมาคมเภสัชวิทยาแห่งประเทศไทย
ภาควิชาเภสัชวิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล

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ออกแบบปก นนทวรรณ ปินตา
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สารบัญ

สารจากคณบดีคณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล

เรียน ประธานคณะกรรมการจัดการประชุมฯ นายกสมาคมเภสัชวิทยาแห่งประเทศไทย
และผู้เข้าร่วมประชุมทุกท่าน

เนื่องในโอกาสที่ภาควิชาเภสัชวิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล ร่วมกับสมาคมเภสัชวิทยาแห่งประเทศไทย ได้ดำเนินการจัดงานประชุมวิชาการประจำปี ครั้งที่ 40 ในหัวข้อเรื่อง “Innovation in Pharmacology” หรือ นวัตกรรมทางเภสัชวิทยา กระผมรู้สึกยินดีที่สมาคมเภสัชวิทยาแห่งประเทศไทยได้จัดงานประชุมวิชาการอย่างต่อเนื่องเป็นประจำทุกปี โดยมีวัตถุประสงค์เพื่อพัฒนาความรู้ทางวิชาการให้ก้าวไกลและทันสมัยอยู่เสมอ โดยเฉพาะหัวข้องานประชุมในปีนี้ ที่มีความสอดคล้องกับแผนยุทธศาสตร์แห่งชาติ ผมมีความเชื่อว่าการพัฒนานวัตกรรมทางเภสัชวิทยาจะมีผลต่อการพัฒนางานวิจัยวิทยาศาสตร์พื้นฐานและต่อยอดไปถึงการรักษาผู้ป่วยเพื่อเพิ่มคุณภาพชีวิตของผู้ป่วยได้ในที่สุด

ปีพุทธศักราช 2561 นี้ จะเป็นปีที่ภาควิชาเภสัชวิทยา ของคณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล ครบรอบ 50ปีการก่อตั้ง และผลิตมหาบัณฑิตและดุษฎีบัณฑิตสาขาเภสัชวิทยา(หลักสูตรนานาชาติ) เป็นแห่งแรกของประเทศไทย ตั้งแต่ปี พ.ศ.2511 โดยตลอดระยะเวลาครึ่งศตวรรษที่ผ่านมา ภาควิชาเภสัชวิทยาได้ผลิตบุคลากรที่มีคุณภาพจำนวนมาก โดยมีศิษย์เก่าทั้งที่เป็นอาจารย์มหาวิทยาลัยและนักวิจัยในภาครัฐและเอกชนทั่วประเทศ

นอกจากนี้ ในปีนี้ยังเป็นโอกาส ครบรอบ 60 ปี แห่งการสถาปนา คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล ซึ่งมีการทำกิจกรรมที่สำคัญต่างๆ รวมถึงการจัดงานประชุมวิชาการประจำปีครั้งนี้ เพื่อเป็นการแสดงให้สังคมเห็นประจักษ์ถึง บทบาทและภาระหน้าที่ของคณะวิทยาศาสตร์ มหาวิทยาลัยมหิดลต่อประเทศไทยและนานาชาติ อีกด้วย

กระผมขออำนวยพรให้การจัดงานประชุมวิชาการของสมาคมเภสัชวิทยาฯ ครั้งนี้ บรรลุวัตถุประสงค์และสำเร็จตามความคาดหมายของการจัดงานทุกประการ บัดนี้ได้เวลาอันสมควรแล้วกระผมขอเปิดงานประชุมวิชาการของสมาคมเภสัชวิทยาแห่งประเทศไทย ครั้งที่ 40 ณ บัดนี้ ขอบคุณครับ

รองศาสตราจารย์ ดร. สิทธิวัฒน์ เลิศศิริ
คณบดี คณะวิทยาศาสตร์
มหาวิทยาลัยมหิดล

สารจากนายกสมาคมเภสัชวิทยาแห่งประเทศไทย

เรียน สมาชิกสมาคมเภสัชวิทยาแห่งประเทศไทย และผู้เข้าร่วมประชุมทุกท่าน

ในนามของสมาคมเภสัชวิทยาแห่งประเทศไทย ขอต้อนรับทุกท่านที่เข้าร่วมประชุมวิชาการประจำปีของสมาคมฯ ครั้งที่ 40 ด้วยความยินดียิ่ง

ในการประชุมวิชาการครั้งนี้ ภาควิชาเภสัชวิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล ได้รับเป็นเจ้าภาพร่วม เนื่องในวาระเฉลิมฉลองการก่อตั้งภาควิชาครบรอบ 50 ปี และในโอกาสครบ 60 ปี แห่งการก่อตั้งคณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล ทางสมาคมฯ ขอแสดงความยินดีต่อสถาบันที่ได้ชื่อว่าผลิตอาจารย์ นักวิจัย นักเภสัชวิทยาให้ประเทศอย่างต่อเนื่องยาวนานมา ณ โอกาสนี้

หัวข้อในการประชุมครั้งนี้ได้แก่ “Innovation in Pharmacology” ซึ่งสอดคล้องกับยุทธศาสตร์ของประเทศโดยใช้นวัตกรรมเพื่อขับเคลื่อนเศรษฐกิจและพัฒนาคุณภาพชีวิตของประชาชน การประชุมในครั้งนี้ได้รับเกียรติจากวิทยากรทั้งในและต่างประเทศ มานำเสนอนวัตกรรมและวิทยาการใหม่ ๆทางเภสัชวิทยา ซึ่งรวมถึงการศึกษาเป้าหมายใหม่ในการออกฤทธิ์ของยา การพัฒนายาใหม่ และการใช้ยาใหม่ นอกเหนือไปจากนี้ทางสมาคมฯ ยังได้รับเกียรติเป็นอย่างสูงจาก ศ.นพ. อุดม คชินทร รัฐมนตรีช่วยว่าการกระทรวงศึกษาธิการ และอดีตอธิการบดีมหาวิทยาลัยมหิดล มาบรรยายในหัวข้อเรื่อง “ยุทธศาสตร์ชาติกับบทบาทของนักเภสัชวิทยา” เพื่อเป็นแนวทางให้นักวิจัยและนักเภสัชวิทยาได้เข้าใจและวางบทบาทให้สอดคล้องกับยุทธศาสตร์ต่อไป

ทางสมาคมฯ ขอขอบพระคุณท่านวิทยากร คณะกรรมการจัดการประชุม รวมทั้งบริษัทห้างร้านต่าง ๆ ที่ให้การสนับสนุนกิจกรรมของสมาคมฯ และขอขอบพระคุณทุกท่านที่เข้าร่วมประชุมในครั้งนี้ กระผมหวังเป็นอย่างยิ่งว่าทุกท่านจะได้รับความรู้ทางวิชาการทันสมัย มีโอกาสพบปะพูดคุยแลกเปลี่ยนความรู้ความคิดเห็น ระหว่างสมาชิกอาวุโส นักศึกษาและนักวิจัยรุ่นใหม่ สร้างกัลยาณมิตรในหมู่นักวิชาการเพื่อสร้างความก้าวหน้าทางเภสัชวิทยาต่อไป

รองศาสตราจารย์ นพ. สุพรชัย กองพัฒนากูล
นายกสมาคมเภสัชวิทยาแห่งประเทศไทย

สารจากประธานจัดการประชุมวิชาการประจำปี ครั้งที่ 40

เรียน คณบดี นายกสมาคมเภสัชวิทยาแห่งประเทศไทย อาจารย์อาวุโส สมาชิกสมาคมเภสัชวิทยาแห่งประเทศไทย และผู้เข้าร่วมประชุมทุกท่าน

ในนามของประธานคณะกรรมการจัดการประชุมร่วมกับสมาคมเภสัชวิทยาแห่งประเทศไทย ขอต้อนรับทุกท่านที่เข้าร่วมประชุมวิชาการประจำปีของสมาคมฯ ครั้งที่ 40 ด้วยความยินดีเป็นอย่างยิ่ง

เนื่องด้วยในปี พุทธศักราช 2561นี้ สมาคมเภสัชวิทยาแห่งประเทศไทยจะมีอายุครบรอบ 40 ปี ซึ่งได้ก่อตั้งขึ้นใน ปีพ .ศ.2521 และ เป็นปีที่ภาควิชาเภสัชวิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล จะมีอายุครบ 50 ปี ในการนี้ในนามของภาควิชาเภสัชวิทยา รู้สึกยินดีและเป็นเกียรติอย่างยิ่งที่ได้รับมอบหมายให้เป็นเจ้าภาพร่วมจัดงานประชุมครั้งนี้จึง งานประชุมจึงจัดขึ้นที่ตึกกลมหรืออาคารเรียนรวมของคณะวิทยาศาสตร์ เพื่อเป็นการคืนสู่เหย้าของชาวศิษย์เก่า และยิ่งกว่านั้นยังสนับสนุน series ของงานวิชาการเพื่อเฉลิมฉลองวาระครบรอบ 60 ปี ของคณะวิทยาศาสตร์ มหาวิทยาลัยมหิดลในปีนี้เช่นกัน งานประชุมวิชาการครั้งนี้จัดขึ้นระหว่างวันที่26-27 เมษายน 2561 ในหัวข้อเรื่อง “Innovation in Pharmacology” หรือนวัตกรรมทางเภสัชวิทยาเพื่อให้สอดคล้องกับนโยบายและยุทธศาสตร์ชาติที่มุ่งเน้นการค้นพบวิทยาการใหม่ ๆ เน้นการนำความรู้มาสร้างสรรค์นวัตกรรมและเพิ่มมูลค่าทรัพยากรที่มีอยู่ในประเทศ โดยเฉพาะการนำสมุนไพรไทยมาใช้ในทางยา ดังนั้นเป้าหมายงานประชุมวิชาการนี้จึงเพื่อส่งเสริมแนวคิดและโอกาสการสร้างนวัตกรรมทางเภสัชวิทยาที่รวมถึง ความคิดสร้างสรรค์ในการค้นคิดสิ่งใหม่ กระบวนการหรือวิธีการใหม่ การพัฒนาสิ่งที่ค้นพบใหม่หรือปรับปรุงสิ่งที่มีอยู่แล้วให้เป็นที่ต้องการด้านการตลาด งานประชุมจึงได้เพิ่ม session ของการประชุมย่อยในวันเสาร์ที่ 28 เมษายน เวลา 9.00 – 12.00 น. สำหรับชาวเภสัชวิทยาและผู้สนใจเพื่อแลกเปลี่ยนความคิดเห็นเพิ่มเติมในประเด็นนวัตกรรมทางเภสัชวิทยา รวมทั้งประเด็นด้านการเรียนการสอนทางเภสัชวิทยาเพื่อรองรับการเปลี่ยนแปลงในความต้องการและความคาดหวังของผู้เรียนในการเข้าเรียนระดับอุดมศึกษาและบัณฑิตศึกษาที่จะเกิดขึ้นในอนาคตอันใกล้นี้

สุดท้ายนี้ขอขอบคุณท่านวิทยากรทุกท่านทั้งชาวไทยและต่างประเทศ ที่ให้เกียรติมาร่วมบรรยายในครั้งนี้ ขอบคุณคณะกรรมการพิจารณาผลงานทางวิชาการ คณะกรรมการตัดสินผลงานทุกท่าน ขอบคุณท่านนายกสมาคมฯและกรรมการสมาคมเภสัชวิทยา ขอขอบคุณทีมงานและบุคลากรภาควิชาเภสัชวิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดลทุกท่านที่ร่วมกันจัดงานครั้งนี้ และขอขอบคุณภาคเอกชนที่สนับสนุนกิจกรรมของสมาคมฯ หวังเป็นอย่างยิ่งว่าผู้เข้าร่วมประชุมทุกท่านจะได้รับความรู้แลกเปลี่ยนความคิดเห็นและมีโอกาสได้ร่วมมือกันในการพัฒนาวงการเภสัชวิทยาให้เข้มแข็งต่อไป

รองศาสตราจารย์ ดร. ดาราวรรณ ปิ่นทอง
ประธานคณะกรรมการจัดงานประชุมวิชาการ

คณะกรรมการจัดการประชุมวิชาการ

การประชุมวิชาการประจำปีครั้งที่ 40 สมาคมเภสัชวิทยาแห่งประเทศไทย
โดยสมาคมเภสัชวิทยาแห่งประเทศไทย ร่วมกับ
ภาควิชาเภสัชวิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล

คณะกรรมการที่ปรึกษา

รองศาสตราจารย์ นพ. สุพรชัย กองพัฒนากูล
รองศาสตราจารย์ ดร. ภญ. ศรีจันทร์ พรเจริญศิลป์
รองศาสตราจารย์ ดร. ภญ. สุพัตรา ศรีไชยรัตน์

คณะกรรมการอำนวยการ

รองศาสตราจารย์ ดร. ดาราวรรณ ปิ่นทอง	ประธาน
รองศาสตราจารย์ ดร. นพวรรณ ภู่มาลา มอราเลส	รองประธาน
รองศาสตราจารย์ ดร.นพ. ณัฐวุธ สิบหมู่	กรรมการ
รองศาสตราจารย์ ดร. ภญ. รัตติมา จีนาพงษา	กรรมการ
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ผู้ช่วยศาสตราจารย์ ดร. ภญ. พรพรรณ วิวิธนาภรณ์	กรรมการ
ผู้ช่วยศาสตราจารย์ ดร. ภญ. วรินกาญจน์ เหมสถาปัตย์	กรรมการ
ผู้ช่วยศาสตราจารย์ ภญ. อรรัตน์ โลหิตนาวี	กรรมการ
อาจารย์ ดร. พญ. พิมทิพย์ สังวรินทะ	กรรมการ
อาจารย์ ดร. นพ. พรรษกร ตันรัตนะ	กรรมการ
อาจารย์ ดร. นพ. สมชาย ญาณโรจนะ	กรรมการ
อาจารย์ ดร. พรทิพา กอประเสริฐถาวร	กรรมการ
อาจารย์ พญ. จิดาภา อาจหาญศิริ	กรรมการ
นางปิยานี รัตนชำนอง	กรรมการ
นางปวีณา ยามานนท์	กรรมการ
นางสาวสุประภา ศรีพระจันทร์	กรรมการ
นางสาวสุวิมล งามผิว	กรรมการ
นางประภา บุญประเสริฐ	กรรมการ
นางศุภสรณ์ แก้วศรี	กรรมการ

คณะกรรมการผู้ทรงคุณวุฒิประเมินผลงาน

รองศาสตราจารย์ ดร. ภญ. ศรีจันทร์ พรเจริญศิลป์
รองศาสตราจารย์ ดร. ภญ. สุพัตรา ศรีไชยรัตน์

ภาควิชาเภสัชวิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล

รองศาสตราจารย์ ดร. นพ. ณัฐวุธ สิบหมู่
รองศาสตราจารย์ ดร. นพวรรณ ภู่มาลา มอราเลส
ผู้ช่วยศาสตราจารย์ ดร. พญ. ณัฏฐิณี จันทรรัตโนทัย
ผู้ช่วยศาสตราจารย์ ดร. ภญ. พรพรรณ วิวิธนาภรณ์
ผู้ช่วยศาสตราจารย์ ดร. ภญ. วรินกาญจน์ เหมสถาปัตย์
อาจารย์ ดร. พญ. พิมทิพย์ สังวรินทะ
อาจารย์ ดร.นพ. กรานต์ สุขนันทร์ธะ
อาจารย์ ดร.นพ. พรรษกร ตันรัตนะ
อาจารย์ ดร. พรทิพา กอประเสริฐถาวร

ภาควิชาเภสัชวิทยา คณะเภสัชศาสตร์ มหาวิทยาลัยมหิดล

ผู้ช่วยศาสตราจารย์ ดร. ภญ. วิลาสินี หิรัญพาณิชย์ ซาโตะ

ภาควิชาเภสัชวิทยา คณะทันตแพทยศาสตร์ มหาวิทยาลัยมหิดล

ผู้ช่วยศาสตราจารย์ ดร. สิรดา ศรีหิรัญ

ภาควิชาเภสัชวิทยา คณะแพทยศาสตร์ศิริราชพยาบาล มหาวิทยาลัยมหิดล

รองศาสตราจารย์ ดร. พญ. อุไรวรรณ พานิช

ภาควิชาเภสัชวิทยา คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

ผู้ช่วยศาสตราจารย์ ดร. วัชรี ลิมปนสิทธิกุล
ผู้ช่วยศาสตราจารย์ ดร. ปิยนุช วงศ์อนันต์
อาจารย์ ดร. พญ. วรรณรัศมี เกตุชาติ

ภาควิชาเภสัชวิทยาและสรีรวิทยา คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

ผู้ช่วยศาสตราจารย์ ดร. ภญ. รัตยา ลือชาพุฒิพร

วิทยาลัยแพทยศาสตร์นานาชาติจุฬาภรณ์ มหาวิทยาลัยธรรมศาสตร์

ศาสตราจารย์ ดร. เกศรา ณ บางช้าง
ผู้ช่วยศาสตราจารย์ ดร. วรรณา ชัยเจริญกุล

คณะเภสัชศาสตร์ มหาวิทยาลัยบูรพา

รองศาสตราจารย์ ดร. มยุรี ตันติสิระ

ภาควิชาเภสัชวิทยา คณะแพทยศาสตร์ มหาวิทยาลัยขอนแก่น

รองศาสตราจารย์ ดร. ลัดดาวัลย์ เส็งกันไพร
ผู้ช่วยศาสตราจารย์ ดร. ศิริมาศ กาญจนวาศ
ผู้ช่วยศาสตราจารย์ ดร. เอื้อมเดือน ประวาฬ

ภาควิชาเภสัชกรรมปฏิบัติ คณะเภสัชศาสตร์ มหาวิทยาลัยนเรศวร

รองศาสตราจารย์ ดร. ภญ. นันทีทิพ ลิ้มเพียรชอบ
รองศาสตราจารย์ ดร. ภญ. รัตติมา จีนาพงษา
ผู้ช่วยศาสตราจารย์ ดร. ภก. มนุพัศ โลหิตนาวี
ผู้ช่วยศาสตราจารย์ ดร. ภก. ณัถวุฒิ แซ่ลิ้ม
ผู้ช่วยศาสตราจารย์ ดร. ภก. ธนศักดิ์ เทียกทอง
ผู้ช่วยศาสตราจารย์ ดร. ภญ. สกลวรรณ ประพฤติบัติ
ผู้ช่วยศาสตราจารย์ ภญ. อรรัตน์ โลหิตนาวี
อาจารย์ ดร. ภญ. จันทิมา เมทนีธร
อาจารย์ ดร. ภญ. วิธู ติลกธรสกุล

ภาควิชาเภสัชวิทยา คณะแพทยศาสตร์ มหาวิทยาลัยศรีนครินทรวิโรฒ

ผู้ช่วยศาสตราจารย์ ดร. ปัทมา ลิ้วนิช
อาจารย์ ดร. ยามาระตี จัยสิน

ภาควิชาเภสัชวิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยสงขลานครินทร์

ผู้ช่วยศาสตราจารย์ ดร. วันดี อุดมอักษร

กำหนดการประชุมวิชาการประจำปีสมาคมเภสัชวิทยาแห่งประเทศไทย ครั้งที่ 40

"นวัตกรรมทางเภสัชวิทยา" (Innovation in Pharmacology)

วันที่ 26-27 เมษายน พ.ศ. 2561 ห้องบรรยายรวม L01 ตึกกลม
และวันที่ 28 เมษายน พ.ศ. 2561 ห้อง Pr. 501 ตึกพีอาร์ ภาควิชาเภสัชวิทยา
ณ คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล พญาไท

วันพฤหัสบดีที่ 26 เมษายน พ.ศ. 2561 ห้องบรรยายรวม L01 ตึกกลม	
08.00-09.00 น.	ลงทะเบียน
09.00-09.30 น.	พิธีเปิดงาน
09.30-10.30 น.	**ปาฐกถาเกียรติยศ รศ. ดร. จิรวัฒก์ สดาวงศ์วิวัฒน์** **Prof. Samuel H.H. Chan** Director, Institute for Translational Research in Biomedicine, Chang Gung Memorial Hospital, Taiwan เรื่อง "*Differential Impacts of Oxidative Stress and Nitrosative Stress on Baroreflex Dysregulation: Clinical and Therapeutic Implications*"
10.00-11.00 น.	พักรับประทานอาหารว่าง
11.00-11.30 น.	**Prof. Ken-ichi Yamada** Faculty of Pharmaceutical Science, Kyushu University, Japan เรื่อง "*Detection and Inhibition of Lipid-derived Radicals*"
11.30-12.30 น.	ประชุมธุรการสมาคมเภสัชวิทยาแห่งประเทศไทย
12.30-13.30 น.	พักรับประทานอาหารกลางวัน
13.30-14.00 น.	**รศ.ดร. ชลภัทร สุขเกษม** คณะแพทยศาสตร์ โรงพยาบาลรามาธิบดี มหาวิทยาลัยมหิดล เรื่อง "*Integrating Pharmacogenomics into Clinical Practice: Promise and Reality*"
14.00-14.30 น.	**รศ.ดร.ศุภโชค มั่งมูล** ภาควิชาเภสัชวิทยา คณะเภสัชศาสตร์ มหาวิทยาลัยมหิดล เรื่อง "*Adenosine Receptor: Therapeutic Target for Treatment of Cardiac Fibrosis*"
14.30-15.00 น.	**รศ. ดร. ณัฐวุธ สิบหมู่** ภาควิชาเภสัชวิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล เรื่อง "*Therapeutic Nitrite*"
15.00-16.30 น.	พักรับประทานอาหารว่าง การนำเสนอผลงานทางวิชาการประเภทโปสเตอร์

วันศุกร์ที่ 27 เมษายน พ.ศ. 2561 ห้องบรรยายรวม L01 ตึกกลม	
08.30-09.00 น.	ลงทะเบียน
09.00-09.45 น.	**ผศ. พญ. ธัญนันท์ เรืองเวทย์วัฒนา** คณะแพทยศาสตร์โรงพยาบาลรามาธิบดี มหาวิทยาลัยมหิดล เรื่อง "*Targeted Therapy: The Novel Therapeutic Strategy in Oncology*"
09.45-10.15 น.	**Dr. Andrew Rowland** Flinders University, South Australia เรื่อง "*Precision Medicine for Targeted Anti-Cancer Medicines*"
10.15-10.45 น.	พักรับประทานอาหารว่าง
10.45-11.15 น.	**เภสัชกรหญิง จิตประภา คนมั่น** คณะแพทยศาสตร์โรงพยาบาลรามาธิบดี มหาวิทยาลัยมหิดล เรื่อง "*Real-Life Experience with TKIs and Immunotherapy: Are There Really Minimal Side Effects?*"
11.15-12.30 น.	**การนำเสนอผลงานทางวิชาการแบบปากเปล่าของนักศึกษา** ประธาน รศ.ดร.ภญ. ศรีจันทร์ พรเจริญศิลป์ รองประธาน ผศ.ดร. ปิยนุช วงศ์อนันต์
	นางสาว กันยารัตน์ แซ่โส ภาควิชาเภสัชวิทยา คณะแพทยศาสตร์ มหาวิทยาลัยขอนแก่น *B01: Genetic polymorphisms of Genes involved in 6-mercaptopurine-induced Hematopoietic Toxicity in Thai children with Acute Lymphoblastic Leukemia*
	นางสาว กนกพรรณ เครือประเสริฐกุล ภาควิชาเภสัชวิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล *B02: Montelukast Induces Triple-negative Breast Cancer Cell Apoptosis via the Reduction of Bcl-2 and p38 MAPK Expression*
	นาย ดิเรก เอกธรรมรัฐ ภาควิชาเภสัชวิทยา คณะแพทยศาสตร์ มหาวิทยาลัยขอนแก่น *A05: Moringa Oleifera* Leaf Extract Promotes Endothelium-Dependent Vasorelaxation by Inhibiting Oxidative Stress and Stimulating the Releases of NO and H_2S
	นาย สราวุฒิ โพธิ์ศรี ภาควิชาเภสัชวิทยา คณะเภสัชศาสตร์ มหาวิทยาลัยมหิดล *A17: Epac is required for Stimulation of A_{2B} Receptor for Inhibition of Endothelin-1-induced Collagen Synthesis and Myofibroblast Differentiation*
12.30-13.30 น.	พักรับประทานอาหารกลางวัน

วันศุกร์ที่ 27 เมษายน พ.ศ. 2561 ห้องบรรยายรวม L01 ตึกกลม	
13.30-14.30 น.	**ศาสตราจารย์คลินิก นพ. อุดม คชินทร** รัฐมนตรีช่วยว่าการกระทรวงศึกษาธิการ เรื่อง "*ยุทธศาสตร์ชาติกับบทบาทของนักเภสัชวิทยา*"
14.30-14.45 น.	พักรับประทานอาหารว่าง
14.45-15.30 น.	**พิธีมอบรางวัลเกียรติยศ ศาสตราจารย์ นพ. อวย เกตุสิงห์** บรรยายพิเศษโดยผู้รับรางวัลเกียรติยศ ผศ. ภญ. ดร. วริษา พงศ์เรขนานนท์ ภาควิชาเภสัชวิทยาและสรีรวิทยา คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
15.30-16.00 น.	การมอบรางวัลนำเสนอผลงานวิจัย พิธีปิดโดยนายกสมาคมเภสัชวิทยาแห่งประเทศไทย

วันเสาร์ที่ 28 เมษายน พ.ศ. 2561 ห้อง Pr. 501 ภาควิชาเภสัชวิทยา	
09.00-12.00 น.	การประชุมเพื่อความร่วมมือทางวิชาการในด้านการวิจัย การสร้างนวัตกรรม และการเรียนการสอนทางเภสัชวิทยาในอนาคต เพื่อส่งเสริมการสร้างนวัตกรรม

ปาฐกถาเกียรติยศ รองศาสตราจารย์ ดร. จิรวัฒก์ สดาวงศ์วิวัฒน์

Assoc. Prof. Chiravat Sadavongvivat Memorial Lecture

Differential Impacts of Oxidative Stress and Nitrosative Stress on Baroreflex Dysregulation: Clinical and Therapeutic Implications

Prof. Samuel H.H. Chan, Ph.D.
Institute for Translational Research in Biomedicine,
Chang Gung Memorial Hospital,
Kaohsiung, Taiwan

The central theme of this lecture is that oxidative stress and nitrosative stress bear differential clinical impact, and that therapeutic intervention is only achievable against pathophysiological, but not pathological conditions. Using as the illustrative example is baroreflex dysregulation, which by itself is not a disease *per se*, although it impacts daily existence of the general populace and in its most severe form is causally related to fatality. Under physiological conditions, the baroreflex provides a rapid negative feedback mechanism that normalizes fluctuations in blood pressure and heart rate induced by environmental insults. Under pathophysiological conditions such as neurogenic hypertension, the baroreflex is rendered dysfunctional because of oxidative stress in its brain stem neural substrates. More importantly, this process is reversible and antioxidant treatment is attainable. When baroreflex is defunct under pathological conditions, nitrosative stress in key nuclei of the baroreflex circuit becomes the primary culprit, which leads to brain dead and other forms of fatality. Intriguingly, this process is irreversible, with diminished therapeutic feasibility. Previous and ongoing work from our group has unveiled an intricate interplay of a multitude of signaling molecules at the level of transcription, translation and post-translational modification in its brain stem neural substrates dictates the phenotypical expression of normal, dysfunctional or defunct baroreflex; and reversible or irreversible disruption of the functional connectivity between key nuclei of the baroreflex circuit determines its pathophysiological or pathological status. Representative examples from our clinical and laboratory work will be used to illustrate these views, including some obtained from recent studies using magnetic resonance imaging/diffusion tensor imaging as an investigative tool in mouse disease models. We conclude that the transition from oxidative stress to nitrosative stress bears crucial clinical impacts on baroreflex dysregulation, and interruption of the associated transition from pathophysiology to pathology should be regarded as a pivotal therapeutic target.

(Supported by the Ministry of Science and Technology and Chang Gung Medical Foundation, Taiwan)

Detection and Inhibition of Lipid-Derived Radicals

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Lipids are easily oxidized by reactive oxygen species, resulting in lipid peroxidation and metabolic products such as lipid-derived electrophiles. These products have been reported to induce inflammation, angiogenesis and ferroptosis. Here, the initial products of lipid oxidation are lipid-derived radicals, the most upstream products that can cause extensive chain reactions affecting lipid peroxidation. Hence, the ability to detect lipid radicals would provide information about one of most important class of molecules causing cellular and tissue damage in a wide range of diseases.

In this symposium, I will present the first fluorescence probe for lipid radicals, NBD-Pen, with high selectivity and sensitivity. To demonstrate bioavailability of NBD-Pen, we tested it in an animal model in which hepatocellular carcinoma was induced by the hepatic procarcinogen diethylnitrosamine (DEN). Furthermore, we found that lipid radical inhibitor significantly relieved inflammation at the early stage of carcinogenesis, as well as following induction of tumor formation by DEN. Thus, we have developed a novel fluorescence probe that provides imaging information about lipid radical generation and potential therapeutic benefits *in vivo*.

Integrating Pharmacogenomics into Clinical Practice: Promise and Reality

Assoc. Prof. Chonlaphat Sukasem, B.Pharm., Ph.D.
Division of Pharmacogenomics and Personalized Medicine,
Department of Pathology, Faculty of Medicine; and
Laboratory for Pharmacogenomics, SomdechPhraDebaratanaMedicalCenter (SDMC),
Ramathibodi Hospital, Mahidol University, Bangkok, Thailand

Currently, several guidelines recommend genetic tests for various *HLA-B* alleles and dosages of drugs that induce SCARs among specific patients carrying risk alleles. Recent studies have documented evidence of several associated polymorphisms in the *HLA-B* allele and SCARs. *HLA-B*57:01, HLA-B*15:02* and *HLA-B*58:01* allele positive individuals have a significantly higher risk of abacavir, carbamazepine and allopurinol hypersensitivity, respectively. With evidence linking association, the Clinical Pharmacogenetics Implementation Consortium (CPIC) dosing guidelines recommend the use of pharmacogenomic tests for presence of the markers before initiating drug therapy in patients. Individuals carrying one or two copies of these markers during clinical genotyping tests presented as “positive”, thus implying a high risk of drug induced- SCARs. Therefore, the drug is not recommended in such patients, as there is high risk of drug-induced SCARs. When there are no copies of the allele detected in genotype tests, a “negative” result is recorded. Patients with negative tests can use the particular drug, according to standard dosing guidelines, because of their lower risk of SCARs related to the use of the drug. To strengthen the utilization of pharmacogenetic testing in our country, we have first invented a low-tech approach and launched “a pharmacogenetic wallet card”. After patients have taken the pharmacogenetic test, their results are entered into the “Pharmacogenetic ID card,” a purple rectangle and plastic wallet size that they can carry around and show to future doctors with their genomic results of those related to the risk of adverse drug reactions or dose recommendation. For example, after patients have taken an HLA test, their results are entered into a plastic “pharmacogenomic wallet card”, which basically contains the genomic results of those related to the risk of SJS/TEN. This card can be carried around and shown to different doctors in the future. Now, the incidence of SJS/TEN has reduced sharply, and now the country is in the phase of eradicating this life-threatening skin rash, in Thailand.

Keywords: drug allergy, SJS/TEN, pharmacogenomics, HLA-B*15:02, HLA-B*58:01, HLA-B*57:01

Adenosine Receptor: Therapeutic Target for Treatment of Cardiac Fibrosis

Assoc. Prof. Supachoke Mangmool, Ph.D.
Department of Pharmacology,
Faculty of Pharmacy, Mahidol University

Cardiac fibrosis is one of the major causes of heart failure and contributes to the abnormality of cardiac functions. After cardiac injury, a number of paracrine and neuronal hormone systems including angiotensin II (Ang II), endothelin-1 (ET-1), and transforming growth factor beta (TGFβ) promote cardiac fibroblast activation, leading to fibroblast proliferation, extracellular matrix (ECM) overproduction, and myofibroblast differentiation. The accumulation of ECM proteins and the differentiation of fibroblast into myofibroblast lead to the replacement of cardiac myocytes with fibrotic scar tissue, resulting in cardiac fibrosis. Ang II and ET-1 act as the profibrotic agents that widely used for a model of cardiac fibrosis. It is crucial to discover the new therapeutic approaches to prevent and reverse underlying cardiac fibrosis induced by Ang II and ET-1.

The previous studies showed that adenosine can reduce the cell proliferation and myofibroblast differentiation of cardiac fibroblasts, supporting the cardioprotective effects of adenosine. Thus, the identification of the molecular mechanisms of adenosine-mediated signaling on inhibition of Ang II- and ET-1-induced cardiac fibrosis will help us to discover the new compounds acting on stimulation of adenosine receptor for prevention of cardiac fibrosis.

Therapeutic nitrite

Assoc. Prof. Nathawut Sibmooh, M.D., Ph.D.
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Inorganic nitrite anion (NO_2^-) is a bioactive nitric oxide (NO) derivative which serves as an endocrine mediator and storage form of NO in many tissues such as muscle, brain, liver, pancreas, and adipose tissue. Most nitrite in blood is produced endogenously from NO synthesized by endothelial nitric oxide synthase; thus its blood levels represent vascular function. Alternatively, it is derived from reduction of nitrate (NO_3^-) obtained from food such as green vegetables. After dietary nitrate intake, nitrate levels increase in plasma and is excreted into saliva where nitrate reductase of oral bacteria reduces salivary nitrate to nitrite. Swallowing saliva increase blood nitrite; thereby contributing to enterosalivary circulation of nitrate-nitrite-NO. Although nitrite is known predominantly as undesired product of NO oxidation with potential carcinogenic effect, accumulating evidences in past 20 years demonstrate physiologic and pharmacologic activities of inorganic nitrite, including vasodilation, antiplatelet, and inhibition of cell death in ischemia-reperfusion injury. In addition, recent epidemiologic studies report no association of dietary nitrite/nitrate intake with cancer.

Blood and tissues contain nitrite at micromolar concentration, and the blood nitrite levels decrease after hypoxia or exercise. Nitrite is converted to NO under acidotic hypoxic condition – a situation in which enzymatic production of NO by nitric oxide synthase is decreased. Thus, nitrite is reserved for non-enzymatic NO production, ensuring adequate NO production in which oxygen-dependent nitric oxide synthase activity is impaired. The reduction of nitrite to NO under this specific situation leads to selectivity of nitrite effect on ischemic tissues with lower systemic adverse effects such as hypotension that may be seen in therapy with NO gas or organic nitrate. Preclinical studies show promising effects of nitrite for therapy of diseases such as myocardial infarction, hypertension, pulmonary hypertension, thromboembolism, sickle cell disease, and peptic ulcer disease. Nitrite has entered phase 2/3 trial for hypertension, pulmonary hypertension, heart failure with preserved ejection fraction, and acute myocardial infarction. Potential therapeutic indications of inorganic nitrite will be presented.

Precision Medicine for Targeted Anti-Cancer Medicines

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Introduction: The implementation of a precision dosing strategy depends on three core elements: (1) the capacity to improve outcomes, (2) the ability to generate high quality evidence of clinical validity, and (3) the practicality of application. In Medical Oncology 'classical' precision dosing strategies such as pharmacogenetics (PGx) and therapeutic drug monitoring (TDM) fail to address these criteria and have struggled to gain traction as actionable clinical strategies.

Aims: To develop novel complementary strategies that facilitate the routine translation of precision dosing for targeted anti-cancer medicines.

Methods: This program of research utilises a continuum of techniques spanning from physiological based-pharmacokinetic (PBPK) modelling and simulation through to healthy volunteer and patient based clinical trials.

Results: Verified PBPK models are used to identify optimal sets of covariates that define variability in exposure. By way of example, 94 % of the variability in steady-state dabrafenib exposure can be defined by considering patient BMI, CYP3A4, CYP2C8 and P-gp protein abundances. In terms of defining *in vivo* drug metabolising enzyme activity, an R^2 of 0.904 was attained for the correlation of circulating CYP3A4 protein expression in exosomes isolated from plasma and midazolam apparent oral clearance (CL/F) in a cohort of 18 to 35 year old healthy Caucasian males of CYP3A4 *1/*1 (wild type) and CYP3A5 *3/*3 (non-functional) genotype pre-and post-rifampicin dosing (300mg QD for 7 days). Furthermore, quantification of the change in exosomal CYP3A4 protein expression defined 92% of the variability in the extent of CYP3A4 induction following treatment with rifampicin.

Discussion: Through the use of novel strategies to identify and quantify biomarkers defining drug exposure, this program is demonstrating the capacity to derive clinically actionable insights from routinely collected samples from pivotal late phase randomised controlled trials regarding the pathways defining drug exposure.

A01 Rimantadine Inhibits Cell Proliferation and Induces Apoptosis of Human Glioblastoma

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Abstract

Amantadine and rimantadine, anti-influenza drugs, have been shown to have efficacy for Parkinson's disease. Amantadine inhibits cell proliferation and induces apoptosis in hepatocellular cancer cell lines. Glioblastoma is the most aggressive brain cancer and standard treatment had failed to improve patient outcome. The present study aims to investigate the anti-proliferative and apoptotic effects of amantadine and rimantadine on A172 and U-87 MG human glioblastoma cells. Cell proliferation and apoptosis was determined with BD Accuri™ C5 flow cytometer. Cells were stained with 5(6)-carboxy-fluorescein diacetate N-succinimidyl ester (CFSE) and Annexin V-FITC and 7-aminoactinomycin D (7-AAD) for cell proliferation and apoptosis assays, respectively. Cells treated with rimantadine at 250 μM showed significantly higher mean fluorescence intensity than mock-treated cells in A172 and U-87 MG cell lines, indicating that rimantadine inhibited cell proliferation. Rimantamine at 500 μM increased percentage of early- and late-stage apoptosis in A172 and U-87 MG cell lines. In contrast, amantadine at 500 μM showed no toxicity on both cell lines. Taken together, the present study demonstrated that rimantadine might represent a novel therapeutic approach for glioblastoma by inhibiting cell proliferation and triggering apoptosis.

Keywords: amantadine, rimantadine, glioblastoma, apoptosis, proliferation

A02 *In vitro* Antimalarial Activity of Laboratory Synthetic Compounds Against *Plasmodium falciparum*

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Abstract

Investigation of new antimalarial agents is urgently required due to emerging drug resistance in malaria parasites. Natural and laboratory synthetic compounds become the major resources in novel antimalarial drug discovery. Chemical structure modification can improve antimalarial potency of original compounds and 4-aminoquinoline pharmacophore is attractive since increasing of chloroquine antimalarial activity has been demonstrated in its derivative compound, piperaquine. The objective of this study was to investigate the antimalarial activity of bicyclic or heterocyclic rings containing compounds against the chloroquine resistant (K1) and chloroquine sensitive (3D7) *Plasmodium falciparum* clones. All compounds in this study were derived from aminoquinoline antimalarial drugs and were synthesized by Dr. Sewan Theeramunkong. *In vitro* antimalarial activity was evaluated based on SYBR green I assay and measured fluorescence intensity of DNA content by multimode microplate reader. Compounds that inhibited parasite growth by 50% (IC_{50}) at concentrations < 1 µM were classified as highly active compounds. Compounds with IC_{50} 1-5 µM were classified as moderately active compounds. Compounds with IC_{50} > 5 µM were classified as weakly active compounds. *In vitro* antimalarial activity of the synthetic compounds correlated between 3D7 and K1 (Pearson correlation (r) = 0.858, p = 0.001). Among eighteen compounds, 6 compounds contained heterocyclic rings and the other 12 compounds contained bicyclic rings. The IC_{50} of bicyclic ring containing compounds ranged between 2.15 and 199.6 µM in 3D7 and 1.92-209.2 µM in K1. The most potent antimalarial activity of the bicyclic compounds was compound **6**, with median IC_{50} (95% CI) in 3D7 and K1 of 2.15 (2.10-2.69) and 1.92 (1.70-2.11) µM, respectively that classified as moderately antimalarial activity. Heterocyclic compounds showed IC_{50} between 6.43 and 162.8 µM in 3D7 and between 6.75 and 424.3 µM in K1. The most potent antimalarial heterocyclic compound was compound **20** with median IC_{50} (95% CI) in 3D7 and K1 of 6.43 (5.80-6.64) µM and 6.75 (6.35-7.50) µM, respectively that classified as weakly antimalarial activity. Although the antimalarial activity of the compounds was still less than chloroquine [IC_{50} (95% CI) in 3D7 was 0.0072 (0.0067-0.0077) µM and that value in K1 was 0.17 (0.15-0.18) µM], the significant correlation of IC_{50} indicated that most compounds, including the two which showed IC_{50} less than 10 µM, exhibited similar antimalarial potency against both chloroquine resistant (K1) and chloroquine sensitive (3D7) *Plasmodium falciparum.*

Keywords: *Plasmodium falciparum*, antimalarial activity, laboratory synthetic compound, aminoquinoline pharmacophore

A03 Nickel Induces Reactive Oxygen Species Production and Cyclooxygenase-2 Expression in Cultured Human Astrocytes

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Abstract

Nickel is a neurotoxic metal known to cause many neurological effects such as lethargy and ataxia. Nickel accumulates in the central nervous system and causes neurotoxicity via many mechanisms including production of reactive oxygen species (ROS). The increased ROS level triggers cyclooxygenase-2 (COX-2) expression, resulting in neuroinflammation. Astrocytes, a major glial cell, play a role in neuronal survival and neuroinflammation. The present study aims to examine the effects of nickel at 50 and 100 µM on cytotoxicity, ROS production and COX-2 expression in human astrocytes. Cell viability, intracellular ROS level and expression of mRNA were measured by MTT, dichlorofluorescein (DCF) and real-time PCR assays, respectively. Data are expressed as mean±SEM. Nickel chloride ($NiCl_2$) up to 50 µM was not toxic to U-87 MG human astrocytoma cells, while $NiCl_2$ at 100 µM reduced cell viability to 76.71 ± 7.11 percent compared to untreated cells at 24 h post-exposure. Consistently, $NiCl_2$ at 50 and 100 µM increased ROS level by 1.17 ± 4.64 and 1.76 ± 0.32 folds compared to untreated cells at 1 h post exposure, respectively. $NiCl_2$ at 50 and 100 µM increased COX-2 mRNA expression by 1.70 ± 0.94 and 7.40 ± 3.63 times compared to untreated cells at 3 h post-exposure, respectively. In conclusion, nickel increases reactive oxygen species and induces COX-2 expression in a dose-dependent manner in human astrocytes.

Keywords: nickel, astrocyte, reactive oxygen species, cyclooxygenase-2

A04 Correlation of T Cell Markers (CD4 and IL-2) in the Treatment of Biopsy-proven Acute Rejection (BPAR) in Tacrolimus-based Therapy Kidney Transplant Patients

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Abstract

Tacrolimus (TAC) is an immunosuppressive agent used for preventing graft rejection. It inhibits T cells activation and reduces immune response. Pharmacokinetic measurements of TAC levels are used for monitor toxicity and serum creatinine (SCr) levels are used for monitoring efficacy. Biopsy-proven acute rejection (BPAR) is the primary efficacy endpoint that predicts kidney graft loss in kidney transplantation. Recent data showed tacrolimus concentration was well correlated with T cell subsets in liver transplantation patients. Pharmacodynamic assay of tacrolimus should be further studied whether it will be used as a new biomarker to monitor kidney transplant patients. The correlation of T cell markers on BPAR treatment needs further investigation in kidney transplantation. This study aimed to investigate the correlation of T cell markers, CD4 and IL-2, on BPAR in tacrolimus-based therapy kidney transplant patients. Whole blood samples of 25 tacrolimus-based therapy kidney transplant patients with BPAR were collected before and at 1 month after BPAR treatment. SCr, TAC trough levels and percentages of T cell markers were recorded and analyzed by flow cytometry at each time point. These BPAR patients showed significant higher percentage of CD4 and IL-2 compared to non-graft rejection patients (72.56% vs 50.26% of CD4 and 32.24% vs 12.92% of IL-2, $p<005$). Moreover, there was a significant decrease of CD4 and IL-2 percentages after BPAR treatment as compared with before BPAR treatment (72.56% vs 45.08% of CD4 and 32.24% vs 18.19% of IL-2, $p<005$). On the other hand, SCr and TAC trough levels were not significantly different before and after treatment. These results indicated that T cell markers showed a significant correlation with BPAR episodes. Percentage of T cell markers may predict the efficacy of BPAR treatment better than SCr and TAC levels. Therefore, T cell markers have the potential to predict efficacy of BPAR treatment and provide a higher sensitivity than only SCr and TAC trough levels in order to identify BPAR episodes in kidney transplant patients.

Keywords: kidney transplantation, kidney transplant patient, biopsy-proven graft rejection, tacrolimus, T cell marker

A05 *Moringa Oleifera* Leaf Extract Promotes Endothelium-Dependent Vasorelaxation by Inhibiting Oxidative Stress and Stimulating the Releases of NO and H_2S

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Abstract

Beneficial effects of *Moringa oleifera* leaf extract have been reported in cardiovascular diseases but little is known regarding its pharmacological effect on vascular function. This study investigated the antihypertensive effect of aqueous extract of *M. oleifera* leaves (MOE) in hypertensive rats and explored its endothelium-dependent vasorelaxant action. Male Wistar rats were given the nitric oxide (NO) synthase inhibitor N^{ω}-nitro-L-arginine-methyl ester (L-NAME, 50 mg/kg/day) in drinking water for 3 weeks to induce hypertension. Systolic blood pressure (SBP) was measured in conscious rats by tail-cuff plethysmography. Vasorelaxant reactivity was determined by measuring the reduction in perfusion pressure in isolated mesenteric arterial beds. Vascular oxidative stress biomarkers including superoxide anion ($O_2^{\bullet-}$) production and malondialdehyde (MDA) levels were determined by lucigenin-enhanced chemiluminescence method and thiobarbituric acid-reactive substance assay, respectively. The results showed that L-NAME administered rats had high SBP, and increased vascular $O_2^{\bullet-}$ and MDA production. In vascular beds of the L-NAME administered rats, acetylcholine-induced endothelium-dependent relaxation was impaired. Interestingly, these abnormalities: the high blood pressure, increased oxidative stress and endothelium dysfunction, were attenuated by concurrent oral MOE (30 and 60 mg/kg/day). Moreover, MOE (0.001-3 mg in 0.1 ml injection volume) caused concentration-dependent relaxation in methoxamine-induced vasoconstriction with a maximum relaxation (R_{max}) of 95±1% achieved at 3 mg. The vasorelaxation produced by MOE was abolished by endothelium denudation (R_{max} = 43±4%) or pretreatment with either NO-synthesis inhibitor L-NAME (R_{max}=72±4%), or hydrogen sulfide (H_2S)-synthesis inhibitor DL-propargylglycine (R_{max} = 83±2%). But, the MOE-induced relaxation was not affected by the cyclooxygenase inhibitor, indomethacin (R_{max} = 93±1%). These results suggest that the antihypertensive effect of MOE may be mediated by inhibition of oxidative stress-induced endothelium dysfunction, and stimulation of endothelium-dependent relaxation of resistance arteries. The endothelium-dependent vasorelaxation in response to MOE is likely mediated by NO and H_2S. MOE has a potential to be developed as a natural product against hypertension.

Keywords: antihypertensive effect, endothelium-dependent vasorelaxation, *Moringa oleifera*

A06 Atractylodin-Loaded PLGA Nanoparticle: Formulation and Characterization

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Abstract

The anti-cholangiocarcinoma activity of atractylodin, a major natural active constituent of the rhizomes of *Atractylodes lancea* (Thunb.) DC, has been demonstrated in a series of studies. However, this compound has poor water solubility and needs to be dissolved in organic solvent. Nanoparticles are now extensively developed for enhancing solubility of hydrophobic compounds in water. The study aimed to formulate atractylodin-loaded poly (lactic-co-glycolic acid) (PLGA) nanoparticles and characterize the prepared nanoparticle formulation. The nanoparticle formulation was developed using solvent displacement method. The encapsulation and loading efficiency were characterized and particle size, and zeta potential were determined by dynamic light scattering technique. Drug release was assessed *in vitro*. The size (mean±SD of diameter) of the prepared atractylodin-loaded PLGA nanoparticles were 161.27±1.87 nm with narrow size distribution (mean±SD PDI: 0.068±0.015) and zeta potential (28.83±0.35 mV). The encapsulation and loading efficiency were 48.31±0.83% and 2.15±0.04%, respectively. Drug release from atractylodin-loaded PLGA nanoparticles was observed up to 87.70±0.47% in 72 hours with biphasic manner. Moreover, the nanoparticles were found to be freely dispersible in water without aggregation. Results suggest that PLGA nanoparticles may be used as an effective drug delivery system for atractylodin. The anti-cholangiocarcinoma activity of this nanoparticle formulation is required.

Keywords: atractylodin, poly (lactic-co-glycolic acid) (PLGA), nanoparticles, solvent displacement method, cholangiocarcinoma

A07 Proteomics Analysis for Identification of Potential Cell Signaling Pathways and Protein Targets of Actions of Atractylodin and β-eudesmol Against Cholangiocarcinoma

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Abstract

Cholangiocarcinoma is the cancer of bile duct with high incidence and mortality worldwide, particularly in Thailand. Clinical efficacy of the currently used chemotherapeutics is limited and there is an urgent need to search for new effective alternative drugs. The aim of the study was to identify potential cell signaling pathways and protein targets of actions of atractylodin and β-eudesmol in cholangiocarcinoma, the two active compounds isolated from *Atracylodes lancea* using proteomics approach. The cholangiocarcinoma cell line, CL-6, was treated with each compound for 3 and 6 hours and the proteins from both intra- and extra-cellular components were extracted. LC-MS/MS was applied following separation of the extract proteins by SDS-PAGE and digestion with trypsin. Signaling pathways and protein expression were analyzed by MASCOT and STITCH software. A total of 4,323 and 4,318 proteins were identified from intra- and extracellular components, respectively. Six and 4 intracellular proteins were linked with the signaling pathways (apoptosis, cell cycle control, and PI3K-AKT) of atractylodin and β-eudesmol, respectively. Four and 3 extracellular proteins were linked with the signaling pathways (NF-κB and PI3K-AKT) of atractylodin and β-eudesmol, respectively. Further study should be performed to confirm the link been the anticholangiocarcinoma activity of both compounds and the expression of the identified proteins involved in these signaling pathways.

Keywords: atractylodin, β-eudesmol, cholangiocarcinoma, proteomics, signaling pathway

A08 Screening of *in vitro* Antimalarial Activity of Crude Ethanolic Extract of Hua-Khao-Yen-Tai (*Smilax* sp.)

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Abstract

Malaria is a mosquito-borne disease caused by the Plasmodium parasite. Re-emerging and spreading of *Plasmodium falciparum* multidrug resistance stains have been a major problem in malaria control. The present study investigated antimalarial activity of the crude ethanolic extract of Hua-Khao-Yen-Tai (*Smilax* sp.) *in vitro.*The rhizomes of Hua-Khao-Yen-Tai, is used in Thai traditional medicine for treatment of inflammation. The phytochemical analysis of crude ethanolic extract was investigated using HPLC. The *in vitro* antimalarial activities of the crude extract were assessed using SYBR green-I assay.The malaria DNA was stained by 1x SYBR green I fluorescence dye and measure fluorescence intensity by microplate reader at the excitation and emission wavelengths of 485 and 530 nm, respectively. Antimalarial acitivity of crude extract against 3D7 (chloroquine-sensitive clone) and K1 (chloroquine-resistant clone) were classified based on their IC_{50} values into six classes as class I, verygood activity (IC_{50}, <0.1 µg/ml); class II, good activity (IC_{50}, 0.1–1.0 µg/ml); class III, good to moderate (IC_{50},>1.0–10 µg/ml); class IV, weak (IC_{50}, >10–25 µg/ml);class V, very weak (IC_{50}, >25–50 µg/ml); and class VI, inactive (IC_{50}, >100 µg/ml). Results revealed that the crude extract contains astilbin flavonols as a major component. The median IC_{50} (concentration that inhibits parasite growth by 50%) values of the extract against 3D7 and K1 were 20.5 and 18.5µg/ml, respectively. However, the IC_{50} standard antimalarial drugs in 3D7 and K1 were 0.00372 and 0.08845µg/ml for chloroquine, 0.01044 and 0.00471 µg/ml for mefloquine and 0.00075 and 0.00056 µg/ml for artesunate, respectively. Results suggest weak antimalarial activity of the crude ethanolic extract of Hua-Khao-Yen-Tai against both *P. falciparum* clones.

Keywords: *Plasmodium falciparum,* Hua-Khao-Yen-Tai, antimalaria activity, *Smilax* sp.

A09 Polymorphism of *ICAM-1* in *Plasmodium falciparum* Isolates in Malaria Endemic Areas along the Thai-Myanmar Border

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Abstract

The intercellular adhesion molecule-1 (ICAM-1) is a cell surface molecule that plays important role in inflammatory response. Significant association between ICAM-1 polymorphism and disease severity and/or treatment response has been demonstrated in several pathological conditions including autoimmune diseases, cardiovascular diseases, cancers, as well as malaria. With regard to malaria, ICAM-1 plays role in cytoadherance of the malaria parasite to human host erythrocytes and cerebral vessels, which can lead to cerebral malaria. Nevertheless, the protective and/or pathogenic effects of ICAM-1 in malaria pathogenesis remain unclear. To clarify this issue, we initially examined the polymorphism of ICAM-1 gene at the position 469 (K469E) in 62 *Plasmodium falciparum* isolates in malaria endemic areas along the Thai-Myanmar border. ICAM-1 polymorphism was characterized by Polymerase Chain Reaction with Restriction Fragment Length Polymorphism (PCR-RFLP) technique using BstuI enzyme. Forty-four isolates (70.97%) were successfully amplified. The K/K variant was detected at highest frequency (34/44, 77.27%), followed by E/K (7/44, 15.91%) and E/E (3/44, 6.82%). More samples will be included in the study to increase statistical power and the association of these gene variants with disease severity and/or treatment outcome will be analyzed.

Keywords: *Plasmodium falciparum*, *ICAM-1*, K469E polymorphisms, malaria, PCR-RFLP

A10 A Systematic Review: Application of *in silico* Models for Antimalarial Drug Discovery

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Abstract

Malaria remains the global public health problem due to the reemergence of drug resistance. There is an urgent need for development of new antimalarial candidates which is effective against resistant malaria parasite. Traditional approach for drug discovery and development is expensive and time-consuming. This systematic review evaluates the published research studies that applied *in silico* modeling during the discovery process of antimalarial drugs. Literature searches were conducted using PubMed, EBSCO, EMBASE, and Web of Science to identify the relevant articles using the search terms "Malaria" "*In silico* model", "Computer-based drug design", "Antimalarial drug", and "Drug discovery". Only the articles published in English between 2008 and May 2015 were included in the analysis. A total of 18 relevant articles met the search criteria. Most articles are studies specific to *Plasmodium falciparum* targets; 3 articles involve target for *P. vivax* and 1 article involves target for liver stage of *Plasmodium*. Both structure-based and ligand-based approaches were applied to obtain lead antimalarial candidates. Two articles also assessed ADMET (absorption, distribution, metabolism, excretion, and toxicity) properties. Confirmation of activity of the candidate leads by *in vitro* and/or *in vivo* assays were reported in some studies. Homology modelling, molecular docking, 2D- or 3D-QSAR and pharmacophore modeling are commonly applied methods. One study used *de novo* synthesis for lead identification and one study applied phylogenetic analysis for target identification/validation. Based on results of the analysis, *in silico* modeling is a useful tool that plays important role in identification/validation of potential antimalarial drug targets and lead candidates during drug discovery phase. Nevertheless, both need to be confirmed by non-clinical (*in vitro* and *in vivo*) and clinical studies.

Keywords: *Plasmodium*, malaria, antimalarial drug, drug discovery, *in silico* modeling, computer-based drug design, systematic review

A11 Genetic Polymorphisms of MRP 1 and MRP 4 in Burmese Patients with *Plasmodium falciparum* Malaria

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Abstract

Malaria remains one of the major threats to human health and WHO concern in most endemic areas. Apart from parasite factor itself, several other factors also contribute to treatment failure. These include pharmacokinetic variability and immune status of each individual patient. Genetic polymorphism of drug transporters is one of the major obstacles for successful treatment outcome. Over expression of drug transporters are also eliminate various useful drugs from the body, causing drug resistance. In this study, we investigated polymorphisms of two multidrug resistance protein (MRP) genes MRP1/ABCC1 and MRP4/ABCC4 in 134 Burmese patients with acute uncomplicated *Plasmodium falciparum* residing in malaria endemic area along the Thai-Myanmar border. Both genes encode transporter proteins which belong to the ATP-binding cassette (ABC) transporters. PCR-RFLP technique was used to identify gene polymorphisms. Data showed that the frequencies of G and C alleles of MRP1 G128C were 0.58 and 0.42, the frequencies of C and T alleles of MRP1 C218T were 0.97 and 0.03, the frequencies of G and A alleles of MRP1 G2168A were 0.95 and 0.05, and the frequencies of G and A alleles of MRP1 G3173A were 0.84 and 0.16, respectively. The frequencies of allele G and A of MRP4 G3724A were 0.69 and 0.31 respectively. Information on MRP1 and MRP4 gene variants in *Plasmodium falciparum* is useful for further analysis of the relation with treatment response with antimalarial drugs in this group of patients.

Keywords: Genetic polymorphism, drug transporter, MRP1, MRP4, *Plasmodium falciparum*

A12 *Atractylodes lancea* (Thunb.) DC. Drug Development for Treatment of Bile Duct Cancer (an *in-vitro* study)

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Abstract

Standard treatment of cholangiocarcinoma (cancer of bile duct) with conventional chemotherapeutic drugs is disappointing with low cure rates. The potential of *Atractylodes lancea* (Thunb.) DC. (AL) for treatment of this type of cancer has previously been demonstrated by our group. The aim of the study was to prepare standardized extract of AL in capsule formulation. Cytotoxic activity of the extracts and 5-fluorouracil (standard control) against CL6 (CCA cell line) and OUMS-36T (normal human embryonic fibroblast cells) was evaluated using standard MTT assay at the concentration range of 0-250 µg/mL. Results showed that, among the three extraction methods (maceration, sonication, and heat-reflux), the maceration method (24 hours, twice) provided the most suitable condition to prepare the extract with regard to the extraction yield (9.65%) and cytotoxic activity (mean±SD IC_{50}: 31.46 ± 2.49 µg/mL). Capsule formulation of the standardized AL extract by maceration method was prepared using ground AL rhizome in 95% ethanol at the ratio of 1:5 (w/v). Lactose (a water-soluble filter) was added to the AL mixture at the ratio 1:3 (w/w). Sodium lauryl sulfate and talcum were selected as suitable surfactant and glidant, respectively. The formulated AL powder possessed good flow ability properties with acceptable weight variation, disintegration and *in vitro* dissolution profiles. The IC_{50} for cytotoxic activity against CL6 cell was 51.80 ± 2.38 µg/mL. Results of the acute and sub-chronic toxicity indicate excellent tolerability profile. Evaluation of stability and chronic toxicity of the capsule formulation are being performed.

Keywords: *Atractylodes lancea* (Thunb.) DC., extracting method, capsule formulation, pharmaceutical properties, acute and subchronic toxicity

A13 Antioxidant Activity of Hot Water Extract of *Momordica cochinchinensis* (Lour.) Spreng. Leaves and Stems

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Abstract

Momordica cochinchinesis (MC) or Gac is an edible plant found in the tropical area of Asia. This plant has various pharmacological actions such as antitumor, antimicrobial and antiinflammation. Our recent studies revealed a high antioxidant activity of the hot water extract of fresh MC leaves and stems. In this study, two hot water extracts of the plant were prepared from fresh MC leaves and stems (FMC) and dried MC leaves and stems (DMC), in order to mimic the methods used during cooking and tea infusion, respectively. The antioxidant activities of the extracts were evaluated by using DPPH, ABTS and H_2O_2 assays. From the DPPH and ABTS assays, the FMC extract (IC_{50s}=19.54 ± 2.00 and 10.83 ± 3.84 µg/mL, respectively) possessed significantly higher antioxidant activities than the DMC extract (IC_{50s}=247.80 ± 35.71 and 51.76 ± 4.57 µg/mL, respectively) (p<0.01). These antioxidant activities of the FMC extract were not statistically different from those of ascorbic acid. However, both FMC and DMC extracts showed relatively low antioxidant activities in the H_2O_2 assay when compared to ascorbic acid with the % inhibitions of 22.73 ± 0.83 and 14.70 ± 0.59, respectively. The amounts of total phenolic content and total flavonoid content of the FMC extract (195.62 ± 5.26 µgGAE/mg extract and 24.11 ± 1.52 µgQE/mg extract, respectively) were higher than those of the DMC extract (55.57 ± 1.29 µgGAE/mg extract and 17.49 ± 0.61 µgQE/mg extract, respectively). The higher antioxidant activities of the FMC extract thus correlated with its total phenolic content and total flavonoid content. These suggested that fresh MC leaves and stems should be used for cooking in order to attain the antioxidant capacity of the plant. Additionally, the leaves and stems of MC can potentially be developed as a food supplement with valuable antioxidant activities in the near future.

Keywords: antioxidant activity, phenolic compounds, flavonoids, *Momordica cochinchinensis*, leaves and stems

A14 Effects of Iron Chelator Deferiprone on Iron Deposition in the Bone of β-Thalassemic Mice

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Abstract

Ineffective erythropoiesis and long-term blood transfusion contribute to iron overload which has been implicated to be a cause of osteoporosis in β-thalassemia patients. Many studies demonstrated the excessive iron deposition in vital organs, such as heart, liver, results in death and various diseases in patients with β-thalassemia. However, study about the iron deposits in the bone of patients with β-thalassemia as well as the ability of deferiprone to remove iron from the bone is unknown. Therefore, the present study aimed to investigate an iron accumulation in both femur and tibia of iron overloaded β-knockout mice (BKO) in comparison with wild-type mice (WT). All mice were loaded with 180 mg iron dextran for 2 weeks. The iron overloaded mice were daily treated with deferiprone for 7 or 21 days. Following euthanasia, vital organs as well as leg bones were collected for analysis. Consistent with previous finding, the levels of iron in vital organs, including liver, kidneys, spleen and heart were significantly increased in iron overloaded mice and these effects were likely to attenuate after treatment with deferiprone. In addition, iron accumulation was also observed in the cell surface of both compact and trabecular bone of iron overloaded BKO and WT mice. However, after treatment with deferiprone for 7 or 21 days, lesser amount of iron appeared in these areas. Taken together, these results suggested that in addition to removal of iron in vital organs, deferiprone may also be effective in removing iron in the compact and trabecular bones.

Keywords: iron overload, osteoporosis, β-thalassemia, deferiprone

A15 Monitoring Hepatic Iron Content in Iron Overloaded β-Thalassemic Mice by Magnetic Resonance Imaging

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Abstract

Increasing of hepatocellular carcinoma in patients with β-thalassemia has been recognized in the past few years. Iron overload is a major cause of this advance complication, therefore monitoring of hepatic iron is necessary for early diagnosis and evaluation of iron chelation therapy. Magnetic resonance imaging (MRI) is known as a useful non-invasive tool for monitoring hepatic iron overload in β-thalassemia patients. However, this technique is limit to a small animal model. This study, MRI technique using T2*value was developed to study hepatic iron content and to monitor the effect of an iron chelator, deferiprone in β-thalassemic mice. Eighteen β-thalassemic mice underwent 1.5 T MRI of the liver and iron level was quantified using T2* value with cardiovascular magnetic resonance (CMR) software. The results showed that the liver T2* values of β-thalassemic mice was 18-37 ms. The values decreased to 3-8 ms in corresponding with increased hepatic iron content (15-20 mg/g liver) in iron loaded β-thalassemic mice ($r=-0.797$, $p<0.001$). Iron chelation with deferiprone (80 mg/g body weight) for 7 days, significantly increased liver T2* value to 11-21 ms. Our results demonstrated that the developed MRI technique using T2* CMR sequence was simple and applicable to a small animal model. This technique may useful to evaluate novel drugs, dosage and regiments for iron chelation therapy.

Keywords: β-thalassemic mice, liver, iron overload, deferiprone, MRI

A16 Development of an HPLC Analysis for Simultaneous Detection of Anti-Tuberculosis Drugs in Localized Drug Release Formulation

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Abstract

Tuberculosis (TB) is a major problem in healthcare systems worldwide. It is caused by *Mycobacterium tuberculosis*. Treatment of tuberculosis typically employs a combination of four first line drugs including isoniazid (INH), rifampin (RIF), pyrazinamide (PYZ) and ethambutol hydrochloride (EMB) to prevent the drug-resistant mutants and recurrence of the disease. Recently, these anti-tuberculosis drugs were formulated as the implantable localized drug release systems for treatment of bone tuberculosis. Here, a reversed phase HPLC method was developed for simultaneous determination of INH, RIF and PYZ in the formulation. Chromatographic system was performed on RIGOL L-3000 Quaternary HPLC Systems, and the separation was carried out on a Mightysil RP-18 GP column (150 × 4.6 mm i.d., 5 µm). The mobile phase was used in gradient conditions comprising acetonitrile (ACN) and 1% v/v ACN in KH_2PO_4 buffer (pH 4.2) at a flow rate of 1.0 ml/min, a total run time of 15 minutes, and a single wavelength UV detection at 254 nm. The concentration range for INH and PZY was 0.5-5 µg/ml while for RIF was 1-10 µg/ml. The retention time of INH, PYZ and RIF were 3.1, 4.4 and 7.7 min, respectively. The developed method showed specificity, linearity ($r^2 > 0.99$), intra-day and inter-day precision (CV < 10%) and accuracy (> 80%). It also has a high sensitivity for TB drugs. The limit of detection and quantification were 0.15 and 0.46 µg/ml for INH, 0.14 and 0.42 µg/ml for PYZ and 0.62 and 1.87 µg/ml for RIF, respectively. This method will be used for determination of amount of drugs in the localized release formulation.

Keywords: HPLC, anti-tuberculosis drugs, isoniazid, pyrazinamide and rifampin

A17 Epac is Required for Stimulation of A_{2B} Receptor for Inhibition of Endothelin-1-induced Collagen Synthesis and Myofibroblast Differentiation

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Abstract

Cardiac fibrosis is manifested by an increase in fibroblast proliferation, overproduction of extracellular matrix proteins, and the formation of myofibroblast that express α-smooth muscle actin (α-SMA). Endothelin-1 (ET-1) is responsible for the pathogenesis of cardiac fibrosis. Overstimulation of endothelin receptors induced cell proliferation, collagen synthesis, and α-SMA expression in cardiac fibroblasts. The favorable regulatory effects of adenosine in the heart, including antifibrotic effects, are mediated through adenosine A_2 receptor (A_2R). After A_2R stimulation, $G_{\alpha s}$ mediates activation of adenylate cyclase (AC), leading to cAMP elevation. cAMP then binds to and stimulates both Protein kinase A (PKA) and exchange factor directly activated by cAMP (Epac), which are important downstream effectors. Although adenosine was shown to exert cardioprotective effects, the molecular mechanisms by which adenosine A_2 receptor inhibit ET-1-induced fibroblast proliferation and α-SMA expression in cardiac fibroblasts remains undefined (A_{2B} receptor mediate). The objective of this study was to determine the mechanisms of cardioprotective effects of adenosine receptor agonist in rat cardiac fibroblast by measurement of mRNA and protein levels of α-SMA. Stimulation of adenosine subtype 2B (A_{2B}) receptor resulted in the inhibition of ET-1-induced α-SMA expression that is dependent on cAMP/Epac/PI3K/Akt signaling pathways in cardiac fibroblasts. Our results confirm a critical role for Epac signaling on A_{2B} receptor-mediated inhibition of ET-1-induced cardiac fibrosis via PI3K and Akt activation. This is the first work reporting a novel signaling pathway for the inhibition of ET-1-induced cardiac fibrosis mediated through the A_{2B} receptor. Thus, A_{2B} receptor agonists represent a promising perspective as therapeutic targets for the prevention of cardiac fibrosis.

Keywords: A_{2B} receptor, cardiac fibrosis, endothelin-1, Epac, α-SMA

A18 Stimulation of A_{2B} Receptor Inhibits Angiotensin II-induced α-SMA Synthesis in Cardiac Fibroblast

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Abstract

Angiotensin II (Ang II) plays a pivotal role in the pathogenesis of cardiac fibrosis. Prolong and overstimulation of angiotensin II type 1 receptor with Ang II-induced collagen synthesis and myofibroblast differentiation in cardiac fibroblasts contribute to cardiac fibrosis. Although adenosine and its analogues are known to have cardioprotective effects, the mechanistic by which adenosine A_2 receptors (A_2Rs) inhibit Ang II-induced cardiac fibrosis has not been definitively established. In the present study, we investigated the effects of exogenous adenosine and endogenous adenosine on Ang II-induced myofibroblast differentiation by determining α-smooth muscle actin (α-SMA) expression and their underlying signal transduction. Elevation of endogenous adenosine levels resulted in the inhibition of Ang II-induced α-SMA synthesis in cardiac fibroblasts. Moreover, treatment with exogenous adenosine which selectively stimulated A_2Rs also suppressed Ang II-induced α-SMA production. These antifibrotic effects of both endogenous and exogenous adenosines are mediated through the A_{2B} receptor (A_{2B}R) subtype. Stimulation of A_{2B}R exhibited antifibrotic effects via the cAMP-dependent and Epac-dependent pathways. Our results establish novel mechanistic insights regarding the role for cAMP and Epac on A_{2B}R mediated antifibrotic effects. Thus, A_{2B}R is one of the promising therapeutic approaches for treatment of cardiac fibrosis.

Keywords: A_{2B} receptor, angiotensin II, endogenous adenosine, collagen, α-SMA

A19 *In Vitro* Antimalarial Activity of Piperine

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Abstract

Malaria is a fatal infectious disease in tropical and subtropical regions especially along the Thailand-Myanmar and Thailand-Cambodia borders. The problem of artemisinin resistance *Plasmodium falciparum* in Southeast Asia is a great concern for disease control and research on discovery and development of new alternative antimalarial drugs is urgently required. In the previous study, the fruits of *Piper chaba* Hunt. was demonstrated to exhibit promising antimalarial activity against asexual stage of both 3D7 and K1 *P. falciparum* clones. The aim of the present study was to further investigate antimalarial activity of piperine, the major isolated constituent of *Piper chaba* Hunt. (fruits) against 3D7 and K1 *P. falciparum* clones. The antimalarial activity determined by using SYBR green-I-based assay and morphological change over 48 hours was observed under light microscope with giemsa staining with 2% parasitemia at ring stage, and 1% hematocrit. The median (range) IC_{50} (concentration that inhibits parasite growth by 50%) values of piperine against 3D7 and K1 were 111.5 (103.3-117.0) and 59 (55.0-70.0) nM, respectively (n=9). A marked change in parasite morphology; slowly grown and the cytoplasm was shrink and condensed compared with untreated control, was observed within 48 hours of piperine exposure. Results of realtime PCR showed no effect of piperine on modulating the expression of the three genes associated with antimalarial drug resistance in *P. falciparum*, i.e., *pfcrt*, *pfmdr1*, and *pfmrp1*. Piperine could be a promising candidate for further development for treatment of malaria based on its antimalarial potency and lower tendency to modulate *P. falciparum* resistant genes and thus, resistance development.

Keywords :piperine, antimalarial drug, *pfmdr 1*, *pfmrp 1*, *pfcrt*

A20 Inhibitory Effect of *Boesenbergia rotunda* Extract on *Candida albicans* Biofilm

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Abstract

Candida albicans, an opportunistic pathogen, is the most prevalent fungal species causing hospital acquired fungal infection. It has ability to adhere on medical devices as well as human tissues and ability to form biofilm as virulence factors. Biofilm plays an important role in forming protective structure thus making it resistant to antifungal drugs. Recently, studies on antifungal effect of *Boesenbergia rotunda* on *C. albicans* have been published, but mechanism of action remains unclear. The aim of this study was to investigate the effects of *B. rotunda* rhizome extract on planktonic growth, adherent stage, biofilm development and biofilm maturity of *C. albicans* (ATCC 10231). The ethanolic extract of *B. rotunda* was examined in the concentration range of 3.125-200 µg/ml, and amphotericin B was used as a positive control. Planktonic growth of *C. albicans*, determined by microdilution method, was not altered when treated with *B. rotunda* extract for 24 hours. The adherent and biofilm stages of *C. albicans* were assessed by MTT assay. The adherence was decreased approximately 20% by the extract. Furthermore, biofilm development was inhibited in a concentration-dependent manner. The results showed half maximal inhibitory concentration (IC_{50}) at 15.6 µg/ml. In mature biofilm stage, it was significantly decreased by *B. rotunda* extract, and the maximum inhibitory effect reached 33.2%. These results suggest that the growth stages of *C. albicans* especially biofilm development could be inhibited by the ethanolic extract of *B. rotunda*. Therefore, *B. rotunda* rhizome might be a potential source of natural compounds with preventive and therapeutic properties against *C. albicans* infection.

Keywords: *Boesenbergia rotunda*, *Candida albicans*, biofilm development, biofilm maturity

A21 Acute Toxicity of Atractylodin and β-eudesmol in Zebrafish Embryos

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Abstract

Atractylodin and β-eudesmol are major active ingredients of the medicinal herb *Atractylodes lancea* (Thnb) DC. (AL). Both exhibited various pharmacological activities including anticancer activity against cholangiocarcinoma (CCA), cancer of the bile duct. Atractylodin and β-eudesmol exerted inhibitory actions on the CCA cell line (CL-6) with mean (±SD) IC_{50} (concentration that inhibits cell growth by 50%) values of 24.0 (±1.98) µM and 21.5 (±2.12) µM, respectively. Despite the widespread use of this plant in traditional medicine in China, Japan, Korea and Thailand, there have been limited studies on its safety and tolerability profile. This current study aimed to evaluate the acute toxicity of atractylodin and β-eudesmol using the well-established zebrafish model. Zebrafish embryos were exposed to a series of concentrations (0, 6.3, 12.5, 25, 50 & 100 µM) of each compound for 72 hours. Atractylodin produced mortality rates of 3.33, 8.33, 15, 88.33, and 100% following exposure to 6.3, 12.5, 25, 50, and 100 µM, respectively, compared to 3.33% in controls. The derived LC_{50} (50% lethal concentration) was 35.90 µM. It produced clear morphological deformities in embryos at higher concentrations. The mortality rates of β-eudesmol following 6.3, 12.5, 25, 50, and 100 µM exposure were 5, 5, 10, 43.33, and 95%, respectively. The derived LC_{50} was 61.53 µM. Morphological deformities followed a similar trend to that of atractylodin. Results suggest that atractylodin and β-eudesmol induce embryonic mortality and malformations in a concentration- and time-dependent manner. Since zebrafish is accepted as a model for humans with very high genetic and phenotypic similarity, the results may imply similar toxicity of both the compounds in humans.

Keywords :atractylodin, β-eudesmol, zebrafish embryos, acute toxicity

A22 Investigation of Pain Behaviour and the Cartilage Regeneration Ability of Adipose-derived Mesenchymal Stem Cells (ASCs) Implantation in a Rat Model of Osteochondral Defect

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Abstract

The management of articular chondral defect remains a challenge owing to its avascular nature. If left untreated this defect may predispose individuals to develop osteoarthritis, in which pain and impairment in joint functionality are known to cause patients to seek for treatment. Recently, there has been a growing interest in the use of adipose-derived mesenchymal stem cells derived from infrapatellar fat pad (IF-ASCs) for the articular cartilage regeneration. However, it is unknown whether these cells can provide pain relief. Due to the superior chondrogenic potential of IF-ASCs, the present study aimed to investigate the potential of using IF-ASCs as a promising source to restore articular cartilage as well as to provide pain relief after implantation into an osteochondral defect of the rat knee. Pain assessment was performed for up to 8 weeks post-implantation by using a weight bearing distribution test. Immunohistological analysis of type II collagen and aggrecan were then assessed to determine the chondrogenic differentiation potential at 4, 8 and 12 weeks post-implantation. During the first two weeks post-implantation, a significant difference in pain associated with osteochondral defect was observed between the untreated defect group and sham-operated group. However, no significant difference in the mean percentage of weight distribution was observed in the implantation groups compared with untreated defect group at any time point. Regarding histological studies, the existence of mature chondrocytes were identified in the implanted site as well as a positively immunostained for type II collagen and aggrecan immunostaining at 4, 8 and 12 weeks, indicating the chondrogenic induction ability of IF-ASCs. These results suggested that pain associated with osteochondral defect can be resolved regardless of IF-ASCs implantation. In addition, the chondrogenic induction ability of IF-ASCs for promoting articular cartilage regeneration was also demonstrated in the rat model of osteochondral defect for up to 12 weeks.

Keywords: osteoarthritis, mesenchymal stem cells, articular cartilage, osteochondral defect

A23 CYP2C9, CYP2C19, CYP2D6, and CYP3A5 Polymorphisms in South East and East Asian Populations: A Systematic Review

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Abstract

Genetic polymorphism is one of the most important factors responsible for inter-individual and inter-ethnic variability in drug response. Studies in major populations like Caucasians, Asians and Africans have provided evidence of differences in the genotype frequencies of major drug metabolizing enzyme cytochrome P450 (CYP). This study aimed to review systematically, all relevant studies that investigated the genetic polymorphisms in CYP2C9, CYP2C19, CYP2D6, and CYP3A5 in South East and East Asian (SEEA) populations. Studies that reported genetic polymorphisms, genotype and allele frequencies in the above genes were retrieved from PubMed database. A total of 86 studies that fulfilled the eligibility criteria representing 16 populations of SEEA, were included in the analysis. The frequency of most common CYP2C9 genotypes *1/*1, *1/*3 and *3/*3 reported in most SEEA populations were comparable. Similarly, comparable frequencies were observed for major CYP3A5 genotypes *1/*1, *1/*3 and *3/*3. Among the 6 major CYP2C19 genotypes, frequencies of *1/*1, *1/*2, *2/*2 and *3/*3 were proportional amongst SEEA while *2/*3 was slightly higher in East Asians and comparably higher frequency of *1/*3 in East asians than Karen and Malay-Indian. Except Singaporeans (Malaya, Chinese and Indian) and Indian-Malaysian, other populations showed relatively similar frequencies for major CYP2D6 genotype *1/*1, *1/*10 and *10/*10 genotype. Abnormal genotype of CYP2C9 is linked to IM (*1/*3), CYP2D6 to *10/*10, CYP2C19 to both PM and IM and CYP3A5 associated with PM (3/*3 and *1/*3). This review indicates that frequency of the major genotypes across SEEA populations are in general comparable. It is noted that more research data are reported from East Asians compared with South East Asians.

Keywords : Genotype frequency, CYP2C19, CYP2C9, CYP2D6, CYP3A5, South East and East Asians

A24 Cytotoxic Activities of the Ethanolic Extract of *Kaempferia galanga* Linn. and its Active Component Against Human Cholangiocarcinoma Cell line and PBMCs

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Abstract

Kaempferia galanga Linn. (KG) is one of the plants in the Zingiberaceae family. Its rhizome extract has been used traditionally in Southeast Asia for analgesic and anti-inflammatory activities. Cholangiocarcinoma (CCA) is a bile duct tumor and which is an important public health problem in the Northeastern region of Thailand. Standard chemotherapeutics for treatment of CCA are currently unsatisfactory. The aim of the study was to investigate cytotoxicity of the ethanolic extract of KG rhizomes including its bioactive compound ethyl-p-methoxycinnamate (EPMC) against the human CCA cell line CL-6 and peripheral blood mononuclear cells (PBMCs) by MTT assay. The half maximal inhibitory concentration (IC_{50}) (mean ± SD) values of KG extract, EPMC, and the reference drug 5-fluorouracil (5-FU) in CL-6 cells were 82.56±24.89, 93.63±22.34 and 19.45±12.37 µg/ml, respectively. The corresponding IC_{50} values for the PBMC cells were 227.87±16.54, 96.14±9.82 and 502.14±11.71µg/ml, respectively. The corresponding selectivity index (SI) values for KG, EPMC and 5-FU compounds on CL-6 as compared to control normal cell line, PBMCs were 5.36, 1.16, and 11.71 respectively. Only KG showed the potency of about 4 times less than of 5-FU. Results provide as a first-step, screening information on potential anti-CCA and cytotoxic effects of the KG extract and EPMC.

Keywords: *Kaempferia galanga* Linn., cytotoxicity, cholangiocarcinoma

A25 Application of Active Targeting Nanoparticles Delivery System for Chemotherapeutic Drugs and Traditional/Herbal Medicines in Cancer Therapy : A Systematic Review

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Abstract

Patients treated with conventional cancer chemotherapy suffer from severe and/or serious side effects of the drugs due to non-selective action of chemotherapeutic drugs to normal cells. Active targeting nanoparticles which are conjugated targeting ligands of cancer cells on the surface of nanoparticles play an important role in improving drug selectivity to the cancer cell. Several chemotherapeutic drugs and traditional or herbal medicines reported for anticancer activities have been investigated for their selective delivery to cancer cells by active targeting nanoparticles. This systematic review summarizes reports on this application. Literature search was conducted through PUBMED database search up to March 2017 using the terms - nanoparticle, chemotherapy, traditional medicine, herbal medicine, natural medicine, natural compound, cancer treatment, and active targeting. Out of 695 published articles, 61 articles with *in vitro* and/or *in vivo* studies were included in the analysis based on the predefined inclusion and exclusion criteria. The targeting ligands conjugated on the surface of nanoparticles included proteins/peptides, hyaluronic acid, folic acid, antibodies/antibody fragments, aptamer, and carbohydrates/polysaccharides. All studies showed that active targeting nanoparticles of chemotherapeutic drugs or traditional/herbal medicines increase selectivity in cellular uptake and/or cytotoxicity. The review suggests several advantages of active targeting nanoparticles over the conventional chemotherapeutic drugs and non-targeted nanoparticle platform, particularly enhancement of drug efficacy and safety. However, clinical studies are required to confirm these findings.

Keywords: drug delivery system, chemotherapy, traditional medicine, cancer treatment, targeted drug delivery

A26 Growth Inhibitory Effect of β-eudesmol on Cholangiocarcinoma Cells is Associated with Suppression of Heme Oxygenase-1 Production and STAT3 Phosphorylation

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Abstract

Cholangiocarcinoma (CCA) is a progressively fatal form of cancer generally arising from malignant transformation of hepatic biliary cholangiocytes. The aim of this study was to investigate *in vitro* growth inhibitory activities of bioactive sesquiterpenoid β-eudesmol (180 µM) in relationship to its underlying potential effects on heme oxygenase-1 (HO-1) production and STAT3 phosphorylation in CCA cells. Human cholangiocarcinoma (CL-6) and normal human embryonic fibroblast (OUMS) cells were used in this study. Cell cytotoxicity was evaluated using MTT assay. Cell culture morphology was visualized using light microscopy. Nuclear morphology was determined using DAPI staining and fluorescence imaging. Anti-proliferative effect was evaluated using colony forming assay. Cell migration was studied using wound healing assay. Relative fold of mRNA expressions were evaluated using real-time PCR. Protein expressions were determined using western blot. β-eudesmol treatment exhibited selective cytotoxicity towards CL-6 as compared to OUMS cells. The compound treatment significantly suppressed colony forming ability of CL-6 cells. In addition, it also induced nuclear fragmentation of CL-6 cells. The compound pretreatment significantly decreased wound healing ability of CL-6 cells in the presence or absence of interlukin-6 stimulation. β-eudesmol treatment significantly suppressed mRNA expression of multiple genes associated with cell proliferation; MCL1, HO-1, MMP-9, NQO1, NRF2 and VEGF-A : 0.5, 0.45, 0.15, 0.4, 0.25 and 0.4 fold changes relative to control. The compound treatment resulted in decreased expression of HO-1 (10 of control) and significantly suppressed STAT3 phosphorylation (29% of control) in CL-6 cells. However, stannous (II) chloride (HO-1 inducer) treatment attenuated cytotoxic effects of β-eudesmol through up-regulation of HO-1 expression. Inhibitory effects of β-eudesmol on HO-1 and STAT3 resulted in stimulation of apoptotic pathway and thus, inhibition of cell proliferation. Taken together, our results suggest that β-eudesmol exerts potent growth inhibitory activity on cholangiocarcinoma cells which might be linked to its inhibitory effect on HO-1 production and STAT3 phosphorylation.

Keywords: β-eudesmol, cholangiocarcinoma, heme oxygenase, STAT3

A27 The *In Vitro* Antiplatelet Aggregation Activity of Deferiprone in Human Platelets

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Abstract

Deferiprone is a successful iron chelator used for the treatment of iron overload conditions. In this study, the activity of deferiprone in platelet aggregation was investigated by using the aggregometer. The in vitro inhibitory activity of deferiprone in normal platelets was determined after adding ADP, collagen, epinephrine and arachidonic acid at concentrations of 4 µM, 2 µg/ml, 1.5 µM and 0.5 mM, respectively. MTT assay was used to find non-toxic concentrations of deferiprone in platelet viability. The results showed that deferiprone inhibited platelet aggregation induced by arachidonic acid or ADP stronger than epinephrine and collagen with the IC50 of 0.24±0.08, 0.25±0.01, 3.36±0.4 and 3.73±0.1 mM, respectively. These results indicated that deferiprone possessed antiplatelet aggregation activity and the site of action may be involved in cyclooxygenase pathways which should be further investigated.

Keywords: deferiprone, antiplatelet aggregation, arachidonic acid, ADP.

A28 Transport Characteristics of Alpha-Mangostin and Gamma-Mangostin from Mangosteen Pericarp Extract Based on hCMEC/D3 Cell Monolayer as a Blood–Brain Barrier Cell Model

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Abstract

Alpha-mangostin and gamma-mangostin, the active compounds extracted from *Garcinia mangostana* L., showed antioxidant and cytoprotective properties. Moreover, alpha-mangostin has neuroprotective effects *in vitro*. In this study, the transport characteristics of alpha- and gamma-mangostin from mangosteen pericarp extract were investigated using hCMEC/D3 cell monolayer as a blood–brain barrier (BBB) *in vitro* cell model. The cytotoxicity of mangosteen pericarp extract on hCMEC/D3 cells was assayed with the MTT method. The contents of two xanthone compounds were determined with high performance liquid chromatography equipped with mass spectrometry (LC-MS/MS). Apparent permeability coefficients (P_{app}) and efflux ratios (PappBL→AP/PappAP→BL) were then calculated to describe the transport characteristics of those two xanthone compounds. The results showed that non-cytotoxic concentrations of mangosteen pericarp extract on hCMEC/D3 cells was around 10 μg/ml. The transport course of both alpha- and gamma-mangostin demonstrated that their transport percentages and transport rates were increased in a nonlinear manner with the time and concentration. The absorption and secretion transport P_{app} values of alpha-mangostin were at the level of 10^{-8} cm/s, while that of gamma-mangostin were at the level of 10^{-9} cm/s. From bidirectional transport assay, both alpha and gamma-mangostin showed efflux ratio less than 2. Therefore, these two xanthone compounds are unlikely to be P-gp substrate.

Keywords :alpha-mangostin, gamma-mangostin, hCMEC/D3 cell lines, transport, apparent permeability coefficients

A29 Alpha-Mangostin Inhibits both Dengue Virus and Cytokine/Chemokine Production

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Abstract

Dengue virus (DENV) infection is a global public health problem. The severity of the disease in patients with dengue infection is correlated with high viral load and excessive immune activation which creates a cascade of cytokine production, called "cytokine storm". Secondary infections are considered a risk factor for developing severe dengue, presumably through a mechanism called antibody-dependent enhancement (ADE). Currently, neither a preventive vaccine nor an effective therapeutic agent is available. Thus, inhibition of DENV and cytokine production could be expected to enhance therapeutic effects for DENV infection. In this work, the effect of α-mangostin (α-MG) from *Garcinia mangostana* Linn. treatment on dengue virus infection and cytokine production in hepatocellular carcinoma HepG2 and U937-derived macrophages was evaluated. α-MG effectively inhibited dengue virus infection in both cell lines. Treatment of dengue infected cells with α-MG (20 μM in 50% ethanol) significantly reduced cell infection rates and viral release into the culture supernatant. Moreover, α-MG effectively reduced cytokine (IL-6 and TNF-α) and chemokine (RANTES, MIP-1β, and IP-10) production in infected HepG2 cells at 24 h post infection. α-MG could also efficiently reduced cytokine (IL-1β, IL-6, and IL-10) and chemokine (IP-10) in ADE-DENV-infected cells. Our results demonstrated that α-MG efficiently inhibits both DENV and cytokine/chemokine production in DENV-infected cells. The combined antiviral and anti-inflammatory properties of α-MG signify the potential of further development as an antiviral chemotherapy to control the spread of the virus and to prevent the complications associated with DENV and immunopathologic responses to DENV infection.

Keywords: dengue virus, α-mangostin, antiviral, anti-inflammatory, cytokine

A30 Effect of *Atractylodes Lancea* (Thunb.) DC. on CYP2A8 Gene Expression in Hamster with *Opisthorchis Viverrini* Induced Cholangiocarcinoma

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Abstract

Atractylodes Lancea (Thunb.) DC. exhibited promising anticancer activities against cholangiocarcinoma. The mechanism of action of *A. lancea* (Thunb.) DC. as an anticancer agent remains to be elucidated. The aim of the present study was to investigate the effect of *A. lancea* (Thunb.) DC. on the expression of cytochrome P450 (CYP) 2A8, an enzyme capable of activating carcinogens including *N*-nitrosodimethylamine, using *Opisthorchis viverrini* (OV)/dimethylnitrosamine (DMN)-induced cholangiocarcinoma in hamsters. The relative expression of CYP2A8 was evaluated by real-time RT-PCR. *A. lancea* extract produced no significant change in CYP2A8 expression compared to untreated control. The results of study have suggested that the mechanism of anticancer effects of *A. lancea* is not mediated via inhibition of CYP2A8. However, the mechanisms of inhibitory effect of *A. lancea* (Thunb.) DC. on other molecular targets in cholangiocarcinoma should be further investigated.

Keywords: cholangiocarcinoma, anticancer, CYP2A8, *Atractylodes lancea* (Thunb.) DC.

A31 Bioactive Constituents Isolated from *Aractylodes lancea* (Thunb) DC. Rhizome Exhibit Synergistic Effect Against Cholangiocarcinoma Cell

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Abstract

Cholangiocarcinoma (CCA) is the major type of bile duct cancer with high morbidity and mortality, particularly in patients with advanced stage. Treatment of CCA remains unsatisfactory due to the lack of sensitive and specific diagnostic tool for early detection as well as effective chemotherapeutics. The aim of the study was to investigate cytotoxic interactions between the three major constituents of the rhizomes of *Atractylodes lancea* (Thunb) DC., *i.e.,* β-eudesmol (BE), atractylodin (AT), and hinesol (HS). Cytotoxic activities against the human CCA cells CL-6 of the dual (BE:AT, BE:HS, and AT:HS) and triple (BE:AT:HS) combinations were evaluated using MTT assay. The cytotoxic interaction of each dual combination was evaluated at five concentration ratios (10:0, 7:3, 5:5, 3:7, and 0:10) using isobologram analysis. For triple combination, the concentration ratio used in the experiment was 1:1.5:2.5 (BE:AT:HS) and analysis of the interaction was performed using polygonogram analysis at the IC_{50} and IC_{90} concentrations (concentration that inhibits cell growth by 50% and 90%, respectively). The BE:AT combination produced additive effect with sum FIC (fractional inhibitory concentration) of 0.967±0.02 (mean±SD). The BE:HS and AT:HS combinations produced synergistic effect with sum FICs of 0.685±0.08 and 0.767±0.09, respectively. The combination of the three compounds produced synergistic interaction with CI (combination index) values of 0.519±0.10 and 0.65±0.17 (mean±SD) at the IC_{50} and IC_{90} concentration levels, respectively. Results obtained would guide further development of AL as potential anti-CCA chemotherapeutics with regard to appropriate pharmaceutical dosage form.

Keywords: cholangiocarcinoma, *Atractylodes lancea* (Thunb) DC., Iiobologram, polyglonogram

A32 Effect of Atractylodin Compound Inhibition on Cholangiocarcinoma Cells Proliferation, Migration, Invasion, and Apoptosis

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Abstract

Cholangiocarcinoma (CCA) is an important cancer in the Great Mekong region, particularly in Thailand. High incidence of CCA is reported from the northeastern Thailand, where the liver fluke *Opisthorchis viverrini* is endemic and that is the major cause of CCA. Limitation of treatment option and the lack of effective diagnostic tool for early detection of CCA are of major concerns for the control of this type of cancer nowadays. The aim of the study was to investigate cytotoxic, apoptotic and inhibitory activities on cell migration and cell invasion of atractylodin, the active constituent of *Atractylodes lancea* (Thunb.) DC. against CCA cell line CL-6. The fibroblast cell (OUMS) was used as normal control cell line. MTT assay was used to test cytotoxicity. CellEvent™ Caspase-3/7 Green Detection Reagent was specific test to analyze activity on cell apoptosis and xCELLigence Real-Time Cell Analyzer (RTCA) DP system was used to test cell migration and invasion. The concentration that inhibits cell growth to 50% (IC_{50}) [median (range) values] for atractylodin in CL-6 and OUMS were 40.03 (39.88-42.39) and 101.74 (99.75-110.42) µg/ml, respectively, with selectivity index of 2.09. The corresponding values for the reference drug 5-fluorouracil (5-FU) were 93.92 (89.74-96.33) and 92.15 (87.48-97.71) µg/ml, respectively, with selectivity index of 2.54. The atractylodin compound also exhibited inhibitory activity on cell migration and cell invasion and induced cell apoptosis in dose dependent manner with the concentrations of 20, 40 and 80 µg/ml. These findings may provide the potential role of atractylodin compound for further development a new approach as chemotherapeutic for CCA.

Keywords: atractylodin, cytotoxicity, cell invasion

A33 *In vitro* Anticancer Effects of Cleistanthin A and Derivatives in Lung Adenocarcinoma Cell A549

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Abstract

The outcome of cancer treatment is limited by drug-resistant population, which causes disease relapse and progression. Finding novel compounds, either from natural or synthetic sources, which can be used as main or adjunctive treatment to chemotherapy, might help improving the treatment outcome. In this study, we investigated the effects of cleistanthin A and its derivatives, MUC 853 and MUC 858, from *Phyllanthus taxodiifolius* Beille, on lung adenocarcinoma A549 cell line. Anticancer effects of cleistanthin A and its derivatives were tested with MTT assay, colony formation assay, and cell cycle analysis. Vehicle-treated samples were used as controls. Treatment of A549 cell line with cleistanthin A and its derivatives resulted in decrease in cell viability in a dose dependent manner, with the IC50 of 33.60 nM for cleistanthin A, 144.49 nM for MUC 853, and 390.38 nM for MUC 858, respectively. The decrease in cell viability by cleistanthin A and its derivatives was accompanied by decrease in colony-forming ability, indicating anti-survival and anti-proliferative effects of the compounds. Moreover, cell cycle analysis revealed that percentage of cells in S phase and G2 phase were decreased, while the cells in G1 phase were increased, suggesting that the anti-proliferative effect of the compounds was exerted trough inhibition of cell cycle progression. More studies on the involved signaling pathway are underway.

Keywords: Cleistanthin A, anticancer, lung adenocarcinoma

B01 Genetic Polymorphisms of Genes Involved in 6-Mercaptopurine-Induced Hematopoietic Toxicity in Thai Children with Acute Lymphoblastic Leukemia

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Abstract

Acute lymphoblastic leukemia (ALL) is a type of cancer in which the malignant cells which large amount of immature lymphocytes result to replace the normal marrow elements. Prolongation of 6-mercaptopurine (6-MP) therapy during a maintenance phase is a key in treatment and this agent may lead to severe hematopoietic toxicity. Inherited deficiency of thiopurine methyltransferase (TPMT) resulting from nonsynonymous polymorphisms is well known exacerbation leads to 6-MP-induced hematopoietic toxicity. However, there have been evidences indicating that patients with wild-type TPMT allele or normal TPMT activity still at higher risk of 6-MP-induced hematopoietic toxicity. Recent genome-wide association studies described a missense variant in the nucleoside diphosphate–linked moiety X-type motif 15 (*NUDT15*) gene that is strongly associated with 6-MP-induced hematopoietic toxicity. This study aims to characterize the polymorphisms of *TPMT*3C*, *NUDT15*3* and *NUDT15*5* genes in Thai children with ALL. Genomic DNAs were isolated from peripheral blood leucocytes. The genotypes of these alleles were determined by TaqMan® SNP genotyping assays. Among 133 subjects, 10 subjects (7.52%) were heterozygous for *TPMT*1/*3C*. Whereas 24 subjects (18.05%) were heterozygous for *NUDT15*1/*3* and 3 subjects were heterozygous for *NUDT15*1/*5* (2.26%). None of the study subjects carried homozygous for *TPMT*3C*, *NUDT15*3* or *NUDT15*5*. The frequencies of variant alleles for *TPMT*3C*, *NUDT15*3* and *NUDT15*5* were 0.04, 0.09 and 0.01, respectively. Results from the present study revealed that the prevalent of polymorphisms in *TPMT* and *NUDT15* genes was common in Thai children with ALL.

Keywords: NUDT15, TPMT, 6-mercaptopurine, acute lymphoblastic leukemia

ความหลากหลายทางพันธุกรรมของยีนที่เกี่ยวข้องกับการเกิดพิษต่อระบบเลือดของยา 6-mercaptopurine ในผู้ป่วยเด็กโรคมะเร็งเม็ดเลือดขาวชาวไทย

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บทคัดย่อ

มะเร็งเม็ดเลือดขาวลิมโฟบลาสติกชนิดเฉียบพลัน เป็นโรคมะเร็งทางโลหิตวิทยาที่เซลล์ต้นกำเนิดเม็ดเลือดในไขกระดูกที่เซลล์ไม่สามารถเจริญเป็นตัวเต็มวัยได้ ส่งผลให้มีการสะสมของเม็ดเลือดขาวตัวอ่อนจำนวนมากไปแทนที่เซลล์ปกติในไขกระดูก 6-mercaptopurine (6-MP) เป็นยาเคมีบำบัดหลักในการรักษาโรคนี้ที่จำเป็นต้องใช้ติดต่อกันเป็นระยะเวลานาน ซึ่งยานี้สามารถทำให้เกิดพิษต่อไขกระดูกอย่างรุนแรงได้ จากรายงานการศึกษาที่ผ่านมาพบว่าความผิดแผกทางพันธุกรรมของยีน Thiopurine methyltransferase (*TPMT*) ในผู้ป่วยแต่ละรายจะส่งผลต่อความสามารถในการทำงานของเอนไซม์ TPMT และการเกิดพิษต่อไขกระดูกของยา 6-MP อย่างไรก็ตามมีรายงานที่พบว่าผู้ป่วยที่มีลักษณะทางพันธุกรรมของยีน *TPMT* ปกติบางรายยังมีความเสี่ยงสูงที่จะเกิดพิษจากยา จากงานวิจัยเชิงจีโนมเมื่อเร็ว ๆ นี้พบว่าความผิดแผกทางพันธุกรรมของยีน nucleoside diphosphate-linked moiety X-type motif 15 (*NUDT15*) สัมพันธ์กับการเกิดพิษของยา 6-MP ด้วย ดังนั้น การวิจัยครั้งนี้จึงมีวัตถุประสงค์เพื่อศึกษาความหลากหลายทางพันธุกรรมของยีน *TPMT*3C*, *NUDT15*3* และ *NUDT15*5* ในผู้ป่วยเด็กโรคมะเร็งเม็ดเลือดขาวชาวไทย โดยทำการสกัด gDNA จากเซลล์เม็ดเลือดขาว เพื่อไปตรวจหาจีโนไทป์ของอัลลีลข้างต้น ด้วยเทคนิค TaqMan SNP genotyping assay ผลการศึกษาพบว่า จากผู้ป่วย 133 ราย มีผู้ป่วย 10 ราย (7.52%) ที่มีจีโนไทป์ แบบ*TPMT*1/*3C* ผู้ป่วย 24 ราย (18.05%) มีจีโนไทป์แบบ *NUDT15*1/*3* ผู้ป่วย 3 ราย (2.26%) มีจีโนไทป์แบบ*NUDT15*1/*5* และพบว่าไม่มีผู้ป่วยรายใดที่มีจีโนไทป์แบบ *TPMT*3C/*3C*, *NUDT15*3/*3* หรือ *NUDT15*5/*5* ความถี่ของ *TPMT*3C*, *NUDT15*3* และ *NUDT15*5* มีค่า 0.04, 0.09 และ 0.01 ตามลำดับ จากการศึกษาครั้งนี้พบว่าอุบัติการณ์ของความผิดแผกทางพันธุกรรมของยีน TPMT และ*NUDT15* พบได้มากในผู้ป่วยเด็กโรคมะเร็งเม็ดเลือดขาวชาวไทย

คำสำคัญ: NUDT15, TPMT, 6-mercaptopurine, มะเร็งเม็ดเลือดขาวลิมโฟบลาสติกชนิดเฉียบพลัน

Introduction

Acute lymphoblastic leukemia (ALL) is a type of cancer in which the malignant cells are lymphoid precursor cells that are arrested in an early stage of development which results in large amount of immature lymphocytes and these lymphoblasts replace the normal marrow elements resulting in a marked decrease in the production of normal blood cells, red blood cells, white blood cells and platelets (1). This may lead to infection, anemia and easy bleeding. This disease is the most common leukemia in children.

6-Mercaptopurine (6-MP) is a common anticancer drug used for treatment of for ALL. This drug is metabolized to the nucleotide intermediates, thioinosine monophosphate (TIMP), by hypoxanthine-guanine phosphoribosyltransferase (HGPRT) and then further metabolized by inosine monophosphate dehydrogenase (IMPDH) and guanosine monophosphate synthetase (GMPS) to 6-thioxanthosine monophosphate (TXMP) and the active metabolites, thioguanine nucleotides (TGN), which are incorporated into DNA (2). Excessive accumulation of a cytotoxic compound TGN, able to induces severe cytotoxicity. On the other hand, 6-MP is mainly detoxified to inactive methylated metabolites, 6-methyl mercaptopurine (6-MMP) by thiopurine methyltransferase (TPMT) and 6-thiouric acid (6-TU) by xanthine oxidase (3).

Inherited deficiency of TPMT resulting from genetic polymorphisms of *TPMT* leads to higher levels of active metabolites of thiopurines and excess hematoxicity during ALL therapy (4). To date, more than 30 allelic variants of *TPMT* have been reported. *TPMT*3A* allele is the most prevalent variant in Caucasians, whereas the *TPMT*3C* allele is the most prevalent variant allele in Asian populations (5). *TPMT*3C* contains a transversion c.719A>G (Y240C) leading to decrease enzyme activity. Individual who carried this variant allele may at higher risk of severe hematotoxicity when treated with 6-MP or 6-MP derivatives (6).

Recent evidences from genome-wide association studies have demonstrated that a missense variant in the nucleoside diphosphate–linked moiety X-type motif 15 (*NUDT15*) gene is strongly associated with 6-MP-induced hematotoxicity. NUDT15 is an enzyme involved in dephosphorylation of 6-MP active metabolites and preventing incorporation of these active metabolites into DNA. Therefore, NUDT15 deficiency resulting from genetic polymorphisms in *NUDT15* is directly resulted in excessive levels of 6-MP active metabolites and increased 6-MP-induced hematopoietic toxicity (7). Two common variant alleles, *NUDT15*3* and *NUDT15*5*, have been identified to be associated with 6-MP-induced hematotoxicity. *NUDT15*3* contains a transition c.415C>T (R139C) while *NUDT15*5* contains a transition c.52G>A (V18I) which resulted in changes in amino acid sequence of the NUDT15 protein and influenced NUDT15 enzyme activity. Recent studies have demonstrated that patients who carried either *NUDT15*3* or *NUDT15*5* alleles were at higher risk of 6-MP-induced hematotoxicity when compared with the patients who carried homozygous *NUDT15*1/*1* (8).

To date, the data on the prevalence of the genes involved in the toxicity of 6-MP in Thai ALL children is still limited. The present study aims to elucidate the genetic polymorphisms of *TPMT* and *NUDT15* in ALL Thai children who received 6-MP treatment.

Materials and Methods

Study population

One hundred and thirty-three Thai children aged between 1-18 years old who were diagnosed for ALL and had been treated with 6-MP according to the Thai Pediatric Oncology Group (Thai-POG) protocol for childhood ALL guidelines chemotherapy in Srinagarind Hospital were recruited. Written informed consent was obtained from subject's parents or guardian after they had been informed both verbally and in writing about the experimental procedures and purposes of the study. Ethical approval for this study was obtained from the Ethical Research Committee of Khon Kaen University.

Detection of NUDT15 and TPMT variants

Blood sample was collected from each subject. Peripheral blood leucocytes were separated and gDNA was purified from peripheral blood leucocytes by using DNA extraction Kit (QIAamp DNA Blood mini kit, Germany). gDNA was used for genotyping of *TPMT*3C* and *NUDT15*3* and *NUDT15*5* polymorphisms by using pre-designed TaqMan® SNP genotyping assays. The Real-Time PCR were performed using a QuantStudio™ 6 Flex Machine (The Applied Biosystems).

Detection of TPMT activity

TPMT activity was determined from patients' red blood cells (RBCs) as previously described (9). In brief, RBCs were washed with ice-cold normal saline solution and then lysed in 0.02 mM potassium phosphate buffer, pH 7.4 by vigorously mixing for 30 sec. TPMT activity in RBC lysates was determined using 6-thioguanine as a substrate and S-adenosyl-L-methionine as a methyl donor. A C18 Nucleosil column and a flow rate of 1.5 mL/min were used instead of a C18 Kingsorb column and a flow rate of 2.0 mL/min. The specific activity of erythrocyte TPMT enzyme was expressed as nmol 6-methylthioguanine (MTG)/g·Hb/h. The hemoglobin content in RBC lysates was determined using an automated hematology analyzer (SysmexXT-2000i, Sysmex America, Inc., Mundelein, Illinois).

Statistical analysis

Genotype and allele frequencies were calculated by counting alleles. Hardy–Weinberg equilibrium was determined by the Chi-squared test. Allele frequencies in different ethnicity from previous studies were compared using the Z-test. A P-value of less than 0.05 was considered statistically significant.

Results

NUDT15 genetic polymorphisms

Of 133 Thai children with ALL who had been treated with 6-MP, 72 males and 61 females with aged 6.99±0.30 (mean ± SEM) were enrolled in this study. Results for *NUDT15* genotypes were shown in Table 1.

Concerning *NUDT15*3*, 109 subjects were homozygous wild-type (81.95%; 95% CI 74.35-88.08), 24 subjects were heterozygous (18.05 %; 95% CI 11.92-25.65). For *NUDT15*5*, 130 subjects were homozygous wild-type (94.74%; 95% CI 93.55-99.53), 3 subjects were heterozygous (2.26%; 95% CI 0.47-6.45). It should be noted that neither homozygous variants of *NUDT15*3* nor *NUDT15*5* were found in the study population.

Table 1. Genotype frequencies of *NUDT15* in Thai ALL patients

Genotype	Number of subject (%)	% Observed genotype frequency [95%CI]	% Expected genotype frequency
NUDT15*3			
**1/*1 (CC)*	109 (81.95)	81.95 [74.35-88.08]	82.81
**1/*3 (CT)*	24 (18.05)	18.05 [11.92-25.65]	16.38
**3/*3 (TT)*	0 (0)	0 (0)	0.81
NUDT15*5			
**1/*1 (GG)*	130 (94.74)	94.74 [93.55-99.53]	98.01
**1/*5 (GA)*	3 (2.26)	2.26 [0.47-6.45]	1.98
**5/*5 (AA)*	0 (0)	0 (0)	0.01

As shown in Table 2, the allele frequencies of *NUDT15*1, *3* and **5* in Thai ALL children were 0.90, 0.09 and 0.01, respectively. Based on the Hardy–Weinberg equilibrium, the genotype frequencies for homozygous wild-type for *NUDT15*3*, heterozygous *NUDT15*1/*3* and homozygous *NUDT15*3/*3* were estimated to be 82.81%, 16.38% and 0.81%, respectively (Table 1). For *NUDT15*5*, the expected genotype frequencies of homozygous wild-type, heterozygous and homozygous variant based on the Hardy–Weinberg equilibrium was 98.01%, 1.98% and 0.01%, respectively (Table 1). The expected genotype frequencies of both *NUDT15*3* and *NUDT15*5* were not statistically different from the actual observed frequencies. Allele frequencies of *NUDT15* alleles in Thai ALL patients are compared with previously reported in various populations (Table 2).

Table 2. Comparative allele frequencies of *NUDT15* in various populations

Gene	Population							
	Thai	Thai	Japanese	Korean	East Asians	Hispanics	Europeans	Lebanese
NUDT15								
**1*	0.899 (0.856-0.932)	0.915	0.800-0.898	0.867-0.896	0.902	0.959*	0.998*	0.996*
**3*	0.090 (0.059-0.131)	0.085	0.102-0.159	0.069-0.104	0.098	0.041*	0.002*	0.004*
**5*	0.011 (0.002-0.033)	ND	0.006	0.011	ND	ND	ND	ND
Ref.	*This study*	(10)	(11), (12)	(13), (14)	(7)	(7)	(7)	(15)

Data in parentheses represent 95% CI, ND; not detected, Different from Thai population (* $P < 0.05$)

TPMT genetic polymorphisms

Among 133 subjects genotyped for *TPMT*, 123 subjects were homozygous for *TPMT*1* (92.5%; 95% CI 86.60-96.34), 10 subjects were heterozygous for *TPMT*1/*3C* (7.5%; 95% CI 3.66-13.39) and none of them carried homozygous for *TPMT*3C*. Based on the Hardy–Weinberg equilibrium, these values were not statistically different from the actual observed frequencies.

Table 3. Genotype frequencies of *TPMT* in Thai ALL patients

Genotype	Number of subject (%)	% Observed genotype frequency [95%CI]	% Expected genotype frequency
TPMT*3C			
**1/*1 (AA)*	123 (92.48)	92.5 [86.60-96.34]	92.63
**1/*3C (AG)*	10 (7.52)	7.5 (3.66-13.39)	7.23
**3C/*3C (GG)*	0 (0)	0 (0)	0.14

The allele frequencies of *TPMT*1* and *TPMT*3C* were 0.96 and 0.04, respectively (Table 4). Allele frequencies of *TPMT* alleles in Thai ALL patients are compared with previously reported in various populations (Table 4).

Table 4. Comparative allele frequencies of *TPMT* in various populations

Gene	Population						
	Thai	Thai	Japanese	East Asians	Hispanics	Europeans	Lebanese
TPMT							
**1*	0.962 (0.932-0.982)	0.947-0.950	0.979-0.978	0.969	0.947-0.964	0.964	0.989
**3C*	0.038 (0.018-0.068)	0.050-0.053	0.013-0.016*	0.031	0.002-0.003*	0.002*	0*
Ref.	*This study*	(5), (16)	(5),(11)	(7)	(5)	(5)	(15)

Data in parentheses represent 95% CI, ND; not detected, Different from Thai population (* $P < 0.05$)

Of 133 all patients enrolled in the study, only 81 blood samples were obtained for determination of TPMT activity. As shown in Figure 1, the mean of erythrocyte TPMT activity of patients with *TPMT*1/*3C* genotype was significantly lower when compared with that of the homozygous wild-type *TPMT*1/*1* genotype (27.36±3.26 vs 42.28±1.6 nmol 6-MTG/g· Hb/h, *P*-value = 0.01).

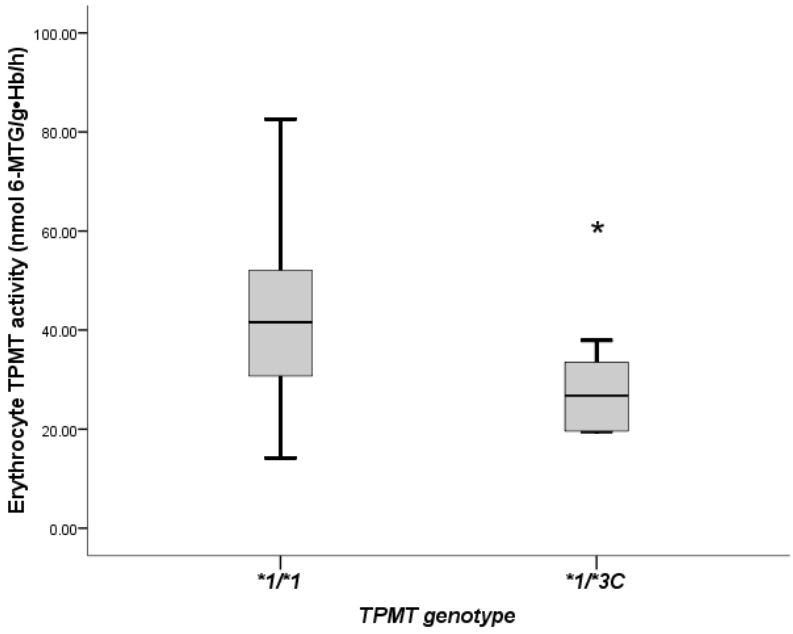

Figure 1. Erythrocyte TPMT activity of Thai ALL patients with homozygous *TPMT*1* (N=75) and heterozygous *TPMT*1/*3C* (N= 6). Lines represent the mean values, the lower and upper borders of the box represent 95% CI. * *P-value* = 0.01

Discussion

Genetic polymorphisms of *TPMT* and *NUDT15* in Thai ALL children are elucidated in the present study. The allele frequencies of *TPMT*3C* in Thai ALL children are significantly higher where as those of *NUDT15*3* and *NUDT15*5* are comparable when compared with other Asian populations. In addition, the allele frequencies of *TPMT*3C*, *NUDT15*3* and *NUDT15*5* in Thai ALL children are significantly higher when compared with Caucasian and Hispanic populations.

The allele frequencies of *NUDT15* variants, *NUDT15*3* and *NUDT15*5*, were 0.09 and 0.01, respectively. These results suggest that *NUDT15*3* allele appears to be a common variant allele in Thai population. Previous studies have demonstrated that 6-MP tolerance was highest in patients with the normal activity NUDT15 (homozygous wild-type) followed by those with intermediate activity (heterozygous variant) and lowest in patients with the low activity (homozygous variant) (17). In addition, variation of *NUDT15* gene is over-represented in Japanese and Korean populations and the genetic polymorphisms of this gene has been suggested as the predominant genetic cause for thiopurine-induced hematotoxicity in Asian populations (17), (18). Moreover, a recent study in Thai ALL children have found an association between *NUDT15*3* and myelosuppression during maintenance therapy with 6-MP and the median dose of 6-MP in patients who carried *NUDT15*3* was significantly lower than those carried homozygous wild-type (10). It should be noted that the allele frequency of *NUDT15*3* found in Thai ALL children is not significantly different from that value found in the present study (0.085 vs 0.090) (Table 2). To our knowledge, the present study is the first report about genetic polymorphism of *NUDT15*5* in a Thai population, whether this variant allele plays a role on hematotoxicity of 6-MP in a Thai population or not needs to be further investigated.

For *TPMT* genetic polymorphism, we have demonstrated relationship between *TPMT* genotype and its enzymatic activity and the results showed that TPMP activity in *TPMT*1/*3C* was about 35% lower than those of homozygous wild-type genotype (Figure 1). These finding consistent with previous report that *TPMT*3C* mutation leads to reduction in enzymatic level and activity (19). The allele frequency of *TPMT*3C* found in Thai ALL children (0.04) trends to be lower than that those previously reported in Thai healthy volunteers (0.05) (5) and Thai ALL children (0.053) (16) (Table 4). Compared with other Asian populations, the frequency of *TPMT*3C* found in the study population was significantly higher than those of Japanese and trend to higher than East Asians suggests that at about 8% of Thai population might have an increased risk of thiopurine-induced toxicity.

Conclusion

In conclusion, results from the present study revealed the allele frequencies of *NUDT15*3*, *NUDT15*5* as well as *TPMT*3C* in Thai ALL children. On comparison with other Asian populations, higher frequency of *TPMT*3C* but comparable frequencies of both *NUDT15*3* and *NUDT15*5* were observed in the study population. Further investigation is needed in order to characterize the relationship between the genetic polymorphisms of these two gene and hematotoxicity caused by 6-MP in Thai ALL children.

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B02 Montelukast Induces Triple-negative Breast Cancer Cell Apoptosis via the Reduction of Bcl-2 and p38 MAPK Expression

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Abstract

Triple-negative breast cancer (TNBC) is the aggressive form of breast cancer and does not response to hormonal or targeted therapy. A previous study reported cytotoxic and antiproliferative effects of two cysteinyl leukotriene receptor antagonists (LTRAs), montelukast and zafirlukast, in MDA-MB-231, a TNBC cell line, at 24 h and 96 h, respectively. However, the mechanisms mediating these effects remain unclear. This study aimed to determine the underlying mechanisms of montelukast's apoptotic effects in MDA-MB-231 cell line. Apoptotic effect of montelukast at 20 µM was determined by annexin V-FITC and 7-Aminoactinomycin D (7-AAD) staining at 6 h, 12 h and 24 h after drug exposure. Flow cytometric analysis showed 20 µM montelukast induced late apoptosis and necrosis at 12 h after exposure. Dichlorofluorescein (DCF) assay showed no change in reactive oxygen species (ROS) level in montelukast-treated cells after 1 and 3 hours of drug exposure. Montelukast decreased the expression of an anti-apoptotic protein, Bcl-2, and p38 MAPK protein at 24 h of treatment. Taken together, the present study demonstrated that montelukast mediated apoptosis of TNBC cells through the downregulation of Bcl-2 and p38 MAPK proteins.

Keywords: triple-negative breast cancer, montelukast, Bcl-2, p38 MAPK

Montelukast เหนี่ยวนำให้เกิดการตายแบบ apoptosis ของเซลล์มะเร็งเต้านมแบบ triple negative โดยลดการแสดงออกของโปรตีน Bcl-2 และ p38 MAPK

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บทคัดย่อ

มะเร็งเต้านมชนิด triple negative มีความรุนแรงและไม่ตอบสนองต่อการรักษาโดยการใช้ฮอร์โมนหรือการให้ยาเจาะจงเซลล์มะเร็ง (targeted therapy) ผลงานวิจัยที่ผ่านมารายงานผลความเป็นพิษต่อเซลล์และฤทธิ์ในการยับยั้งการแบ่งตัวของ cysteinyl leukotriene receptor antagonist 2 ชนิด ได้แก่ montelukast และ zafirlukast ในเซลล์ MDA-MB-231 ซึ่งเป็นเซลล์มะเร็งเต้านมแบบ triple negative ที่เวลา 24 และ 96 ชั่วโมง ตามลำดับ อย่างไรก็ตามกลไกที่เหนี่ยวนำให้เกิดผลเหล่านี้ยังไม่แน่ชัด การศึกษาในครั้งนี้มีเป้าหมายที่จะค้นหากลไกที่เกี่ยวข้องกับการตายของเซลล์แบบ apoptosis ที่เกิดจากยา montelukast ในเซลล์ MDA-MB-231 การศึกษา ผลของ montelukast ขนาด 20 μM ที่มีต่อการตายแบบ apoptosis ที่เวลา 6, 12 และ 24 ชั่วโมง ใช้การย้อม annexin V-FITC และ 7-Aminoactinomycin D (7-AAD) การประมวลผลโดยใช้ flow cytometry พบว่า 20 μM montelukast ชักนำให้เกิดการตายของเซลล์แบบ late apoptosis และ necrosis ที่ 12 ชั่วโมงหลังจากได้รับสาร การวัดระดับสารอนุมูลอิสระ (reactive oxygen species) โดยวิธี dichlorofluorescein (DCF) ไม่พบความเปลี่ยนแปลงระดับในการสร้างสารอนุมูลอิสระในเซลล์ที่ได้รับ montelukast เป็นเวลา 1 และ 3 ชั่วโมง ในขณะที่ montelukast มีผลในการลดการแสดงออกของโปรตีนที่ยับยั้งการตายของเซลล์ชื่อ Bcl-2 และ p38 MAPK การศึกษาในครั้งนี้แสดงให้เห็นว่า montelukast ควบคุมให้เกิดการตายของเซลล์มะเร็งเต้านมแบบ triple negative โดยลดการแสดงออกของโปรตีน Bcl-2 และ p38 MAPK

คำสำคัญ: เซลล์มะเร็งเต้านมชนิด triple negative, montelukast, Bcl-2, p38 MAPK

Introduction

Breast cancer is the most common cancer in women worldwide (1). The incidence of breast cancer in Thai women increased 3.1% per year from 2000 to 2012 (2). The progression and the response to treatment of breast cancer is based on the expression of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2). The standard treatments for patients, who do not express all three biomarkers (triple-negative breast cancer, TNBC), are surgery and classical chemotherapy drugs such as cyclophosphamide, methotrexate and fluorouracil (3). There is currently no specific treatment for TNBC; therefore, the identification of novel therapy and understanding how these drugs work is needed.

Leukotrienes (LTs) play an important role in the pathogenesis of asthma, inflammatory diseases and immediate hypersensitivity reaction (4). In asthma patients, LTRAs users shows the decreased risk of breast, lung, colorectal and liver cancers compared with non-users (5). The activation of cysteinyl leukotriene receptor 1 (CysLT1R) in colon cancer cells and intestinal epithelial cells increases survival and proliferation, the two important steps in tumorigenicity (6). The overexpression of CysLT1R also increases the risk of cancer-induced death and reduced survival in breast cancer patients (7). We recently reported that cysteinyl leukotriene receptor antagonists (LTRAs), montelukast and zafirlukast at 20 μM, reduced cell viability and induced apoptosis of MDA-MB-231, a TNBC cell line, at 24 hours (8). However, mechanisms responsible for apoptotic effect of LTRAs remain unclear.

The present study aims to determine the effects of time on apoptotic effects of montelukast and investigate its underlying mechanisms in MDA-MB-231. The apoptotic effects of montelukast were measured at 6 h, 12 h and 24 h after drug exposure using annexin V-FITC and 7-aminoactinomycin D (7-AAD) staining and analyzed via flow cytometry. Reactive oxygen species (ROS) level was detected using dichlorofluorescein (DCF) assay and the expression of an anti-apoptotic protein, Bcl-2, and p38 MAPK protein were determined using western blotting.

Materials and Methods

Cell line and culture condition

MDA-MB-231 cells were obtained from American Type Culture Collection (ATCC) and cultured in Dulbecco's Modified Eagle *Medium (DMEM, Gibco, USA)* with 1% penicillin/streptomycin (Merck, Germany) and 10% fetal bovine serum (Gibco, USA). Cells were maintained at 37 ºC in humidified atmosphere of 5% CO_2.

Drugs

Montelukast was purchased from Sigma (USA) and dissolved in dimethyl sulfoxide (DMSO, Sigma, USA). The final concentration of DMSO is 0.04% (v/v) in culture media.

Apoptotic assay

Cells were plated on 6-well plates. At 60-70% confluency, cells were treated with 20 μM montelukast or DMSO. After 6, 12 and 24 h, cells were harvested and incubated in dark with annexin V-FITC (BD Pharmingen™, United States) and 7-aminoactinomycin D (7-AAD) (BD Pharmingen™, United States). Cells positive for annexin V-FITC without or with 7-AAD staining were considered as early and late apoptotic cells, respectively. Cells positive

for 7-AAD without annexin V-FITC staining were considered as necrotic cells. Apoptotic cells were analyzed using a BD Accuri™ C6 flow cytometer (BD Bioscience, United States) with a minimum of 5,000 events from each experiment. Data were expressed as the percentage of cell population.

Reactive oxygen species detection

Cells were plated on 96-well black plates with clear bottom in DMEM without phenol red. At 60-70% confluency, cells were pre-incubated with dichloro-dihydro-fluorescein diacetate (DCFH-DA, Invitrogen, USA) for 40 min, and subsequently washed and treated with 20 µM montelukast, DMSO or *tert*-Butyl hydroperoxide (tBuOOH, Sigma, USA). The fluorescence intensity was measured at 1 and 3 h post-exposure by a microplate reader (Varioskan Flash, Thermo Scientific, USA) with excitation and emission spectra of 490 nm and 535 nm, respectively. DCF fluorescence intensity in DMEM without phenol red-treated cells was defined as 100% in each experiment.

Western blotting

Cells were plated on 60-mm dishes and treated with 20 µM montelukast or DMSO at 60-70% confluency. After 24 h, cells were washed in cold phosphate buffered saline (PBS) and lysed in lysis buffer (50 mM Tris, 150 mM NaCl, and 0.5% nonidet P-40, pH 7.4) with *protease inhibitor cocktails* (Calbiochem, Germany). Equal amount of proteins was loaded into SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Biorad, United States). Membranes were probed with primary antibodies for Bcl-2, p38 and β-tubulin (Cell Signaling, USA) followed by HRP-labeled anti-rabbit or anti-mouse IgG secondary antibodies (Jackson ImmunoResearch, USA). Immunoreactive bands were visualized using Clarity™ ECL Western Blotting substrate (Bio-Rad, United States) on UltraCruz® autoradiography film (Santa Cruz Biotechnology, USA). The band intensity of proteins of interest was measured and normalized to expression of a housekeeping protein, β-tubulin.

Statistical analysis

Data were presented as the mean±SEM ($N \geq 3$). The experiments were analyzed by one-way analysis of variance (ANOVA) with Dunnett's post-hoc test or two-way ANOVA with Bonferroni post-tests using GraphPad® Prism, version 5.0 (GraphPad software Inc., San Diego, CA).

Results

Montelukast caused morphological change and induced apoptosis and necrosis in MDA-MB-231 cells

Our previous study indicated that 20 µM montelukast resulted in more than 90% apoptotic cells at 24 h post-exposure (8). In the present study, MDA-MB-231 cells were treated with 20 µM montelukast or DMSO for 6, 12 and 24 h. At 6 h post-exposure, montelukast-treated cells showed the retraction of process. At 12 h, cells became round and the reduction of cell density was observed. At 24 h, all cells became round and detached from surface (Figure 1). Next, we investigated whether montelukast induced apoptosis and/or necrosis using annexin V-FITC and 7-AAD staining and flow cytometry. The percentage of cells positive for annexin V-FITC without or with 7-AAD staining represents early and late apoptosis, respectively. The percentage of cells positive for 7-AAD without annexin V-FITC was considered as necrotic cells. Representative dot plots showed the montelukast increased late apoptotic cells and necrotic cells with increasing time (Figure 2). Quantitative analysis

showed that montelukast induced late apoptosis and necrosis at 12 and 24 h post-exposure (Figure 3) while there was no increase in early apoptosis.

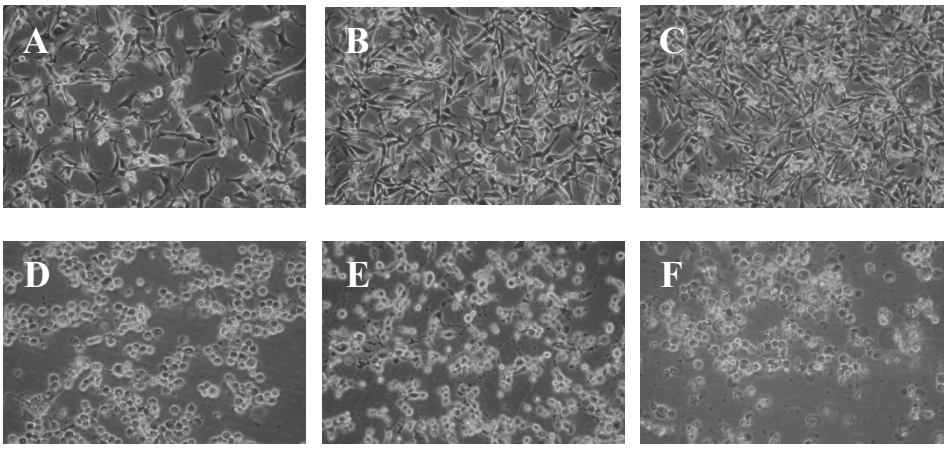

Figure 1. Effects of montelukast on morphology and density of MDA-MB-231 cells. DMSO-treated cells at 6 h (A), 12 h (B) and 24 h (C) and montelukast-treated cells at 6 h (D), 12 h (E) and 24 h (F) were observed using an inverted light microscope (10x objective). At all time points, DMSO did not alter cell shape and cell density increased over time, whereas montelukast-treated cells showed cell shrinkage since 6 h and floating cells were observed at 24 h post-exposure.

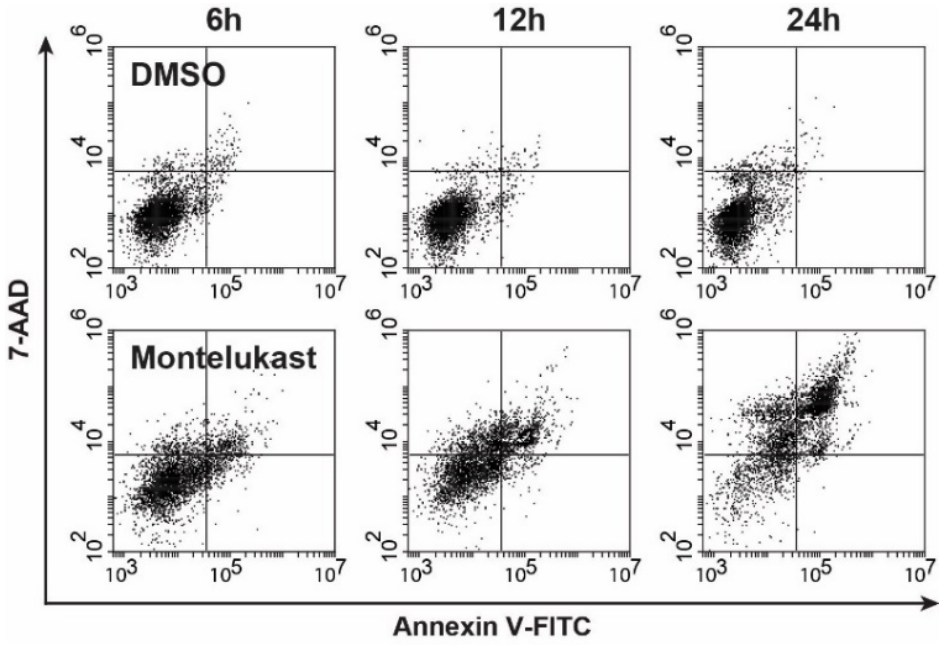

Figure 2. Flow cytometry dot plots of DMSO and montelukast-treated cells at 6 h, 12 h and 24 h of drug exposure. MDA-MB-231 cells were stained with annexin V-FITC and 7-AAD and analyzed by a flow cytometer. Increased levels of annexin V and 7-AAD positive cells were found at 12 and 24 h post-exposure**.**

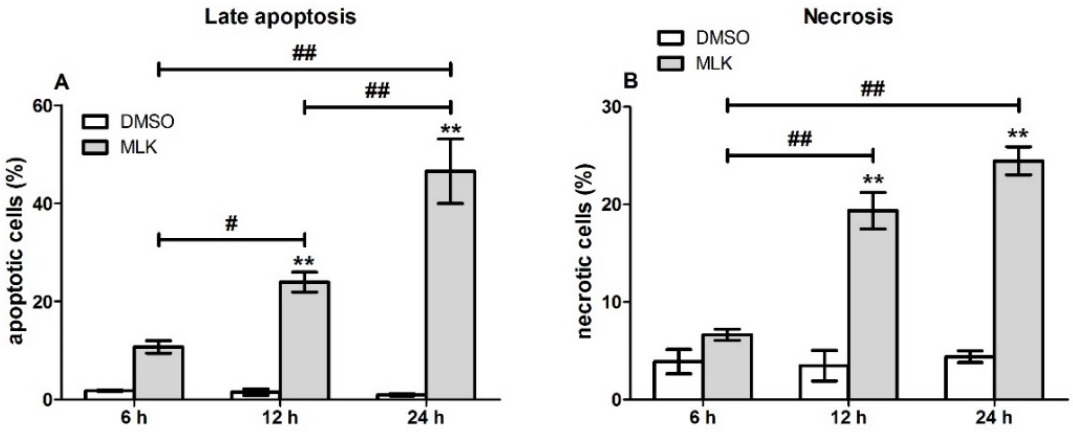

Figure 3. Montelukast induced late apoptosis and necrosis in a time-dependent manner. Percentage of late apoptotic cells (A) and necrotic cells (B) were measured at 6, 12 and 24 h after exposure to either DMSO or 20 μM montelukast (MLK) (N=4). Data were presented as mean ± SEM. Two-way ANOVA was used for statistical analysis and statistical significance was donated as **($p < 0.01$) in comparison between DMSO-treated cells and montelukast-treated cells at the same time point and #($p < 0.05$) and ##($p < 0.01$) in comparison among montelukast-treated cells from different time points.

Montelukast did not increase ROS generation in MDA-MB-231 cells

To examine whether apoptotic effects of montelukast was mediated by oxidative stress, ROS generation was measured at 1 and 3 h post-exposure using DCF assays. Cells treated with DMSO and *tert*-Butyl hydroperoxide (tBuOOH) were used as a mock control and a positive control, respectively. ROS levels were shown as the percentage of DCF fluorescence compared to untreated cells. The results showed no change on the ROS generation in montelukast-treated cells compared to DMSO-treated cells in both times of exposure (Figure 4).

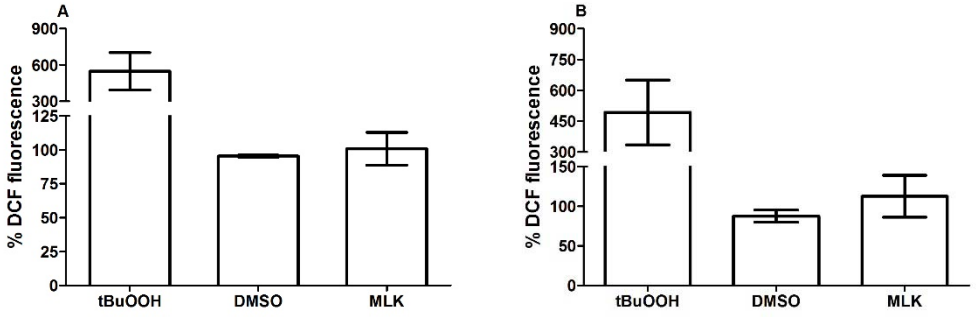

Figure 4. Montelukast did not induce ROS generation. ROS levels were measured by DCF assays after 1 h (A) and 3 h (B) post-exposure to 20 μM montelukast (MLK). There was no significant difference in ROS generation between montelukast-treated cells and DMSO-treated cells (N=3). Data were presented as mean ± SEM. One-way ANOVA was used for statistical analysis.

Montelukast reduced the expression of Bcl-2 and p38 MAPK proteins in MDA-MB-231 cells

Next, we examined the effects of montelukast on apoptotic and MAPK pathways using western blotting. The results showed the decreased expression of Bcl-2 and p38 MAPK proteins in montelukast-treated cells at 24 h of treatment (Figure 5).

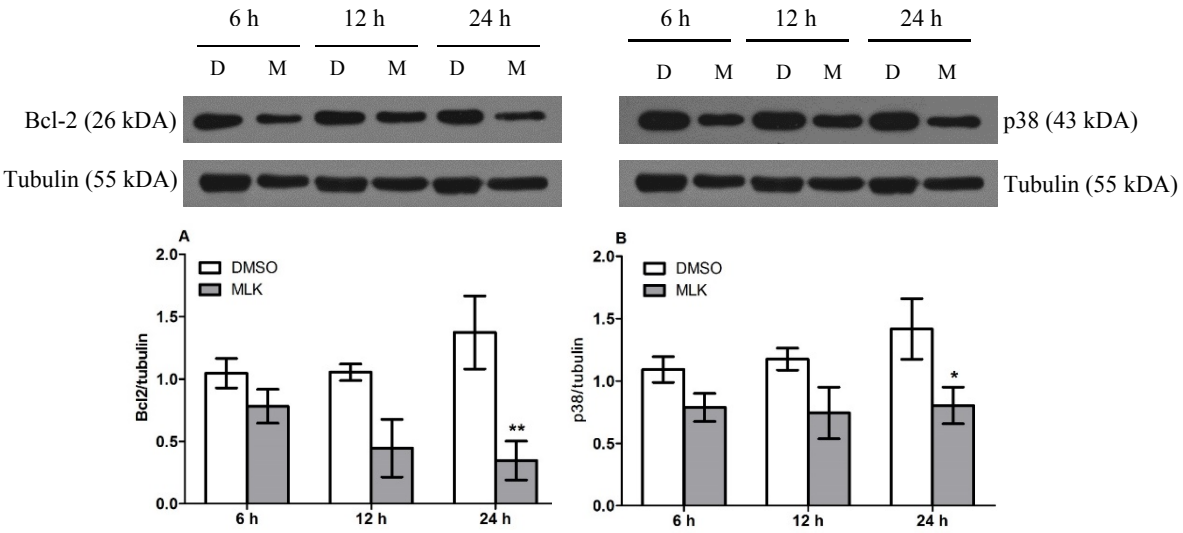

Figure 5. Montelukast (MLK) decreased expression of Bcl-2 and p38 MAPK proteins. Expression of Bcl-2 (A) and p38 MAPK (B) at 24 h post-exposure was decreased (N=4). Data were presented as mean±SEM. Two-way ANOVA was used for statistical analysis and statistical significance was donated as *($p < 0.05$) and **($p < 0.01$) in comparison between DMSO-treated cells and montelukast-treated cells. (D = DMSO, M = montelukast)

Discussion

Montelukast was reported to decrease the breast cancer risk in asthma patients (5). Our previous study reported that montelukast and zafirlukast decreased cell viability, induced apoptosis and inhibited proliferation of MDA-MB-231 cell line with median toxic concentration (TC_{50}) between 5-10 µM (8). The present study demonstrated that cytotoxic dose of montelukast (20 µM) induced apoptosis through downregulation of Bcl-2 and p38 MAPK proteins.

The cytotoxic effects of montelukast were studied in several types of cancers. In lung cancer cells, 50 µM montelukast showed apoptotic effect at 12 h post-exposure (9). At 24 h of drug exposure, 25 µM montelukast induced human colon carcinoma cell apoptosis (10) and 100 µM montelukast induced apoptosis in prostate cancer cells and renal cell carcinoma (11, 12) . The previous study reported cytotoxic effects of montelukast in TNBC cells at 24 h post exposure (8). In this study, 20 µM montelukast induced apoptosis of TNBC cells at 12 h post-exposure and showed greater effect at 24 h post-exposure, indicating that the apoptotic effect was time-dependent.

Several anticancer drugs induced oxidative stress, leading to cell death (13). Low concentrations of ROS promote cancer cell survival, while high concentrations leads to

oxidative stress and apoptosis (14). This study found no change in ROS generation in montelukast-treated cells compared to a mock control both 1 and 3 h drug exposure, demonstrating that montelukast-induced apoptosis was not mediated by the direct ROS generation. However, montelukast may alter the expression of oxidative stress-related gene.

LTRAs induce apoptosis of lung cancer cells (9) and brain tissue of rat and mice (15, 16) via downregulation of Bcl-2 protein. Bcl-2 is considered a key anti-apoptotic protein because it inhibits the release of cytochrome c and apoptosis-inducing factors that directly activate caspases (17). In the present study, montelukast-treated cells showed the reduction of Bcl-2 protein expression at 24 h, suggesting that montelukast mediated apoptosis by decreasing Bcl-2 expression.

p38 MAPK plays a role in either the regulation of cell death or the stimulation of cell survival depending on stimuli and cell types (18). In myeloid cell 1, p38 inhibitors decreased Egr2 expression, resulting in the reduction of Bcl-2 expression (19). The present study found low p38 MAPK expression in montelukast-treated cells together with the reduction of Bcl-2 expression, suggesting that the low levels of Bcl-2 might be mediated by p38. However, further studies are needed to confirm that montelukast decreases Bcl-2 expression through p38 pathway.

Conclusion

Montelukast induced late apoptosis and necrosis on breast cancer cells in a time dependent manner. The apoptotic effects of montelukast on apoptosis are mediated by the decrease in an anti-apoptotic protein, Bcl-2, and p38 MAPK protein expression, but not via the direct ROS generation. However, further studies on other apoptotic-related proteins such as Bax, Bak and caspases as well as other proteins in MAPK pathways would provide more information on how LTRAs trigger apoptosis.

Acknowledgements

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B03 Increased Expressions of Nitric Oxide Synthase and Guanylate Cyclase and Correlation with Tumor Progression in Human Breast Cancer

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Abstract

The nitric oxide (NO) at low concentrations promote proliferation, angiogenesis and invasiveness of cancer cells through the NO/sGC/cGMP pathway. By western blot analysis, we investigated expressions of endothelial nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS), and soluble guanylate cyclase β1 subunit (sGCβ1) in breast cancer tissues from patients (N = 20). Correlation of the eNOS, iNOS and sGCβ1expressions with clinical data was examined. We showed breast cancer expressed significantly higher levels of eNOS, iNOS and sGCβ1 than normal adjacent tissues ($P<0.05$). The eNOS expression was positively correlated with tumor size ($r=0.5178$, $P=0.0194$). The expression of iNOS was significantly higher in invasive (grade I/II and grade III) than in non-invasive breast cancer. Moreover, sGCβ1 expression was higher in patients with lymph node metastasis than patients without lymph node metastasis. In conclusion, breast cancer tissues exhibit higher protein expression in NO pathway including eNOS, iNOS, and sGCβ1, which are correlated with tumor progression.

Keywords: eNOS, iNOS, sGCβ1, breast cancer

การแสดงออกของไนตริกออกไซด์และ Guanylate Cyclase ที่เพิ่มขึ้นและความสัมพันธ์กับความรุนแรงของเนื้องอกในมะเร็งเต้านม

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บทคัดย่อ

ไนตริกออกไซด์ (NO) ที่ความเข้มข้นต่ำช่วยส่งเสริมการเพิ่มจำนวนเซลล์ การสร้างเส้นเลือดใหม่และการบุกรุกของเซลล์มะเร็งผ่านทาง NO / sGC / cGMP จากการศึกษาโดยใช้ western blot เราได้วัดการแสดงออกของเอนไซม์ endothelial nitric oxide synthase (eNOS), เอนไซม์ inducible nitric oxide synthase (iNOS) และเอนไซม์ guanylate cyclase β1 (sGCβ1) ในเนื้อเยื่อมะเร็งเต้านมจากผู้ป่วย (N=20) และมีการศึกษาความสัมพันธ์ระหว่างการแสดงออกของโปรตีน eNOS, iNOS และ sGCβ1 กับข้อมูลทางคลินิก การศึกษาของเราพบว่าเนื้องอกมะเร็งเต้านมมีระดับ eNOS, iNOS และ sGCβ1 สูงกว่าเนื้อเยื่อปกติที่อยู่ติดกันอย่างมีนัยสำคัญ ($P<0.05$) การแสดงออกของ eNOS มีความสัมพันธ์เชิงบวกกับขนาดเนื้องอก ($r=0.5178$, $P=0.0194$) การแสดงออกของ iNOS สูงขึ้นอย่างมีนัยสำคัญทางสถิติในมะเร็งเต้านมที่มีการรุกราน (grade I / II และ grade III) มากกว่ามะเร็งเต้านมที่ไม่รุกราน นอกจากนี้การแสดงออกของ sGCβ1 สูงกว่าในผู้ป่วยมะเร็งเต้านมชนิดที่รุกรานไปต่อมน้ำเหลืองมากกว่าชนิดที่ไม่รุกราน สรุปได้ว่าเนื้อเยื่อมะเร็งเต้านมมีระดับโปรตีน eNOS, iNOS และ sGCβ1 สูงขึ้นซึ่งมีความสัมพันธ์กับความก้าวหน้าของเนื้องอก

คำสำคัญ: eNOS, iNOS, sGCβ1, breast cancer

Introduction

Breast cancer as the leading cause of cancer death among women around the world, there were about 14.9 million new cases around the world in 2012 (1). Nitric oxide (NO), a short-lived endogenously produced gas, acts as a diffusible messenger in intercellular communication and intracellular signaling, to regulate various physiological processes (2, 3). NO can be rapidly oxidized to nitrite (NO_2^-) and nitrate (NO_3^-) after being synthesized from L-arginine and oxygen (O_2) by the enzyme nitric oxide synthase (NOS) (4). Both endothelial NOS (eNOS or NOS3) and neuronal NOS (nNOS or NOS1) have a constitutive expression, whereas inducible NOS (iNOS or NOS2) is Ca^{2+}-independent, elicited by inflammatory mediators (5). Soluble guanylate cyclase (sGC) is an intracellular enzyme acting as NO receptors. The binding of NO to β1 subunits of sGC leads to enzyme dimerization that converts guanosine triphosphate (GTP) into cyclic guanosine monophosphate (cGMP). This second messenger cGMP can regulate physiological functions, including vasodilation, inhibition of platelet aggregation, and neurotransmission (6).

Low NO concentrations (<100 nM) produced from eNOS promote proliferation, angiogenesis, and invasiveness of cancer cells through the NO/sGC/cGMP and nitrosative pathways (7). From previous immunohistochemistry (IHC) study on NOS expression in human breast cancer tissues, iNOS and eNOS expressions increased in invasive carcinomas in comparison to in situ carcinomas. Ductal carcinomas had significantly more eNOS positive compared with the lobular or tubular carcinoma subtypes (8). Another study demonstrated a gradual increase in iNOS from low-grade in situ carcinomas to high-grade invasive ductal breast carcinomas (9). However, there were some conflicting results from different studies on sGCβ1 in breast cancer cells. One study demonstrated that activation of NO-cGMP-protein kinase G (PKG) pathway inhibited growth and induced apoptosis of MDA-MB-468 and SK-Br-3 breast cancer cell line, which were related with reduced expression of both sGC subunits. Down-regulation of sGC in breast tumor may thus protect breast cancer cells from the growth inhibition induced by NO-cGMP (10). In contrast, high sGCβ1 protein expression levels were observed in MDA-MB-468 breast cancer cells and these levels were correlated with the sGC activity and a marked increase in cGMP levels upon exposure to the combination of a NO donor and a sGC activator (11).

In this study, we aimed to determine iNOS, eNOS, and sGCβ1 protein expression levels in the breast cancer tissues from patients, and their correlation with clinical features of breast cancer patients.

Materials and Methods

Tissue collection from patients

Human breast tissue samples were obtained from the Department of Surgery, Faculty of Medicine Ramathibodi Hospital, Mahidol University, Bangkok, Thailand. Breast tissues were collected from operation room and stored at -80°C until use (N=20). This study was approved by Committee on Human Rights Related to Research Involving Human Subjects, Faculty of Medicine Ramathibodi Hospital, Mahidol University based on the declaration of Helsinki, protocol ID 03-58-32.

Protein extraction from human breast tissue

50 mg of tissues were lysed in 1 mL of tissue lysis buffer, containing 150 mM sodium chloride, 50 mM Tris, PH 7.4, 1.0% NP-40, 0.1% sodium dodecyl sulfate (SDS), and the

proteinase inhibitor cocktail. Tissues were kept on the ice and disrupted using a tissue homogenizer (Polytron PT 3100). Protein lysates stored at -80°C until use.

Measurement of eNOS, iNOS, and sGCβ1 protein expression levels in breast tissue

eNOS, iNOS and sGCβ1 protein expression levels were measured by western blot. The concentration of protein was determined by bicinchoninic acid (BCA) protein assays. Equal amount of protein (25 μg) was loaded into well of 10% SDS-PAGE and then proteins were transferred to nitrocellulose membranes. Blots were blocked with non-fat milk in Tris-buffered saline containing 0.1% Tween-20 overnight at 4°C and incubated with anti-eNOS (BD Biosciences, San Jose, CA, USA), anti-iNOS (Santa Cruz Biotechnology, Santa Cruz, CA), anti-sGC β1 (Cayman Chemical, Ann Arbor, MI, USA), and anti-β-actin (Cell Signaling Technology) overnight at 4°C. Band density was visualized using horseradish peroxidase (HRP) conjugated secondary antibodies and enhanced chemiluminescent (ECL) substrate on film. Band density was determined using ImageJ software.

Statistical analysis

The results were presented as median with interquartile range (IQR) or mean±SD, depending on data normality. Comparison was performed by Mann-Whitney or Kruskal–Wallis test. The Wilcoxon paired-test was used to compare the difference between matched samples. All data were analyzed using GraphPad Prism software (Version 6.0).

Results

A total of 20 female patients with breast cancer samples were recruited in this study. The characteristics of patients are shown in Table1. The mean age at diagnosis was 52.85 years old. The histological types consist of 4 non-invasive carcinomas, 1 invasive solid papilary carcinoma, and 15 invasive ductal carcinomas. Approximately half of patients had lymph node metastasis. The hormone receptor status was positive in 16 patients.

Table 1. Patients characteristics

Number of patients	20
Age at diagnosis (in years), mean±SD	52.85±10.61
Tumor size in cm, median (min, max)	2.75 (0.2, 8.5)
Histological types	
Non-invasive	4
Invasive grade I	1
Invasive grade II	9
Invasive grade III	6
Lymph node	
No metastasis	9
Metastasis	11
Hormone receptor status (estrogen and progesterone receptors)	
Positive	16
Negative	4

The eNOS, iNOS and sGCβ1 protein expression levels in human breast tissue

Levels of eNOS, iNOS and sGCβ1 protein expression were determined by western blot. Expression of the protein of interest was normalized to housekeeping protein, β-actin, and presented as relative fold change. The results showed that breast cancer expressed significant higher levels of eNOS, iNOS and sGCβ1 than in adjacent normal tissues (Figure 1).

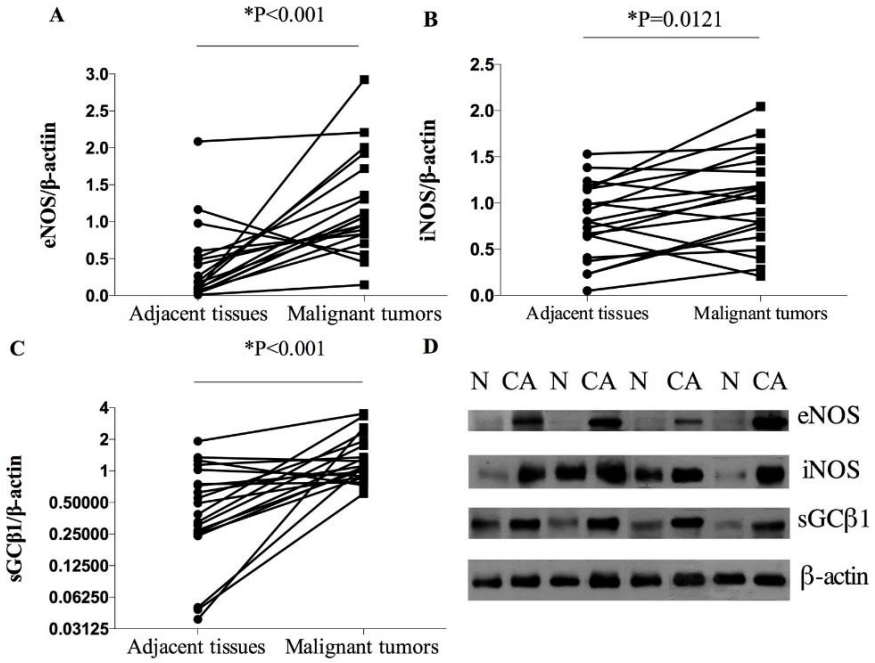

Figure 1. Expressions of eNOS (A), iNOS (B), and sGCβ1 (C) in normal adjacent and breast cancer tissues from 20 patients. Comparison was done by Wilcoxon signed rank test. (D) The representative western blots of eNOS, iNOS, sGCβ1 and β-actin in 4 pairs of adjacent non-tumor tissues (N) and matched breast cancer (CA). β-actin was used as a loading control.

Correlation of eNOS, iNOS, and sGCβ1 expression with clinical features

The Spearman rank correlation test revealed a significant positive correlation between eNOS and tumor size (r = 0.5178, P=0.0184) (Figure 2). Patients with larger tumor sizes were more likely to express higher eNOS levels. The iNOS expression level was significantly different among patient with three invasive types of cancer. Patients with grade III invasiveness cancer exhibited the highest level of iNOS expression, followed by patients with grade I-II and non-invasive cancer (P=0.026, Kruskal–Wallis H-test) (Figure 3). Breast cancer from patients with lymph node metastases expressed higher levels of sGCβ1 than those without lymph node metastases (P=0.042, Mann-Whitney test) (Figure 4). However, no association was found between expressions of eNOS, iNOS and sGCβ1, and hormone receptor status.

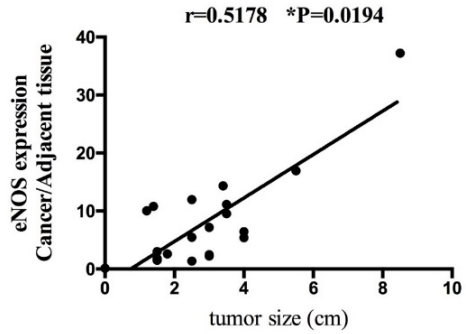

Figure 2. Correlation between eNOS expression and tumor size. Spearman rank correlation coefficient r = 0.5889, P=0.0194.

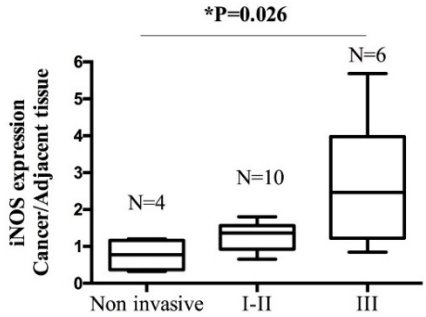

Figure 3. Box and whisker plot of iNOS expression in breast cancer tissues at different invasive grade. Boxes indicate the interquartile range (IQR). Horizontal bars in boxes indicate the median. Comparison was done by Kruskal–Wallis test.

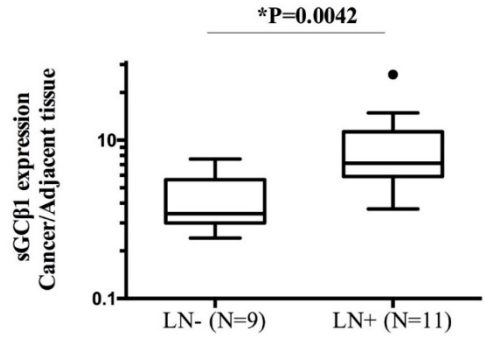

Figure 4. Box and whisker plot of sGCβ1 expression in breast cancer tissues of patients without lymph node metastasis (LN-) and with lymph node metastasis (LN+). Boxes indicate the interquartile range (IQR). Horizontal bars within boxes indicate the median. Dot represents outlier. Comparison was done by Mann-Whitney test.

Discussion

Previous immunohistochemistry studies demonstrated eNOS and iNOS predominately expressed in the cytoplasm of human breast cancer cells (12, 13). Similarly, we found breast cancer tissues expressed higher levels of eNOS and iNOS than the normal adjacent tissues. The positive association between eNOS expression and the positive estrogen receptors status

was found in previous study (12). However, due to limited number of breast cancer with negative hormone receptors (N = 4) in our study, we could not do comparison analysis with positive hormone receptor cancer.

NO is important for cancer progression. First, we found there was positive correlation between eNOS expression and tumor size, although there was no correlation in previous study (14). Vascular endothelial growth factor (VEGF) are associated with increased eNOS activity and inhibition of apoptosis in human breast carcinoma xenografts (15). eNOS-derived NO can stimulate VEGF expression in breast cancer cells (16). The positive correlation between eNOS expression and tumor size suggest NO is essential for tumor growth, probably via increased expression of VEGF and other growth factors. Second, we found that invasive cancer has higher iNOS expression than non-invasive cancer, suggesting the role of iNOS in invasiveness. This finding is consistent with a previous study showing that invasive carcinoma has a higher level of iNOS expression than in situ carcinoma (9).

The sGCβ1 expression was higher in breast cancer with lymph node metastasis than that without lymph node metastasis, suggesting the role of scGCb1 in invasiveness. The sGCβ1 mRNA expression of MDA-MB-468 (human triple negative breast cancer cell line) was 35-fold greater than DU-145 control cells (11). NO could induce C3L5 murine mammary tumor cell migration through sGC and ERK1/2 pathway (17). Moreover, the NO/ sGCβ1/ cGMP pathway has been identified as an important mediator of VEGF effects on lymphatic vessel function in inflammation (18). Herein, sGCβ1 expression might be involved in mediating tumor-associated lymph angiogenesis and metastasis to the lymph nodes. However, the distribution of eNOS, iNOS and sGCβ1 protein in breast cancer tissues need further immunohistochemistry study.

Conclusion

eNOS, iNOS and sGCβ1 expressions increased in breast cancer tissues compared with normal adjacent tissues. eNOS expression was correlated with tumor size. Higher iNOS expression was found in invasive cancer. Increased sGCβ1 expression was found in breast cancer with lymph node metastasis. The NOS/NO/sGCβ1 pathway may mediate breast cancer cell progression in human breast cancer tissue.

Acknowledgements

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B04 Elevation of 5-Lipoxygenase and Cysteinyl Leukotriene Receptor Type 1 and Reductionof Cysteinyl Leukotriene Receptor Type 2 expression in Meningiomas

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Abstract

Meningiomas are the second most common types of primary brain tumors. Current chemotherapy drugs are not very effective and there is no standard treatment or biomarker for meningiomas. Leukotrienes are proinflammatory lipid mediators that play a role in the pathophysiology of asthma and cancers. A recent large epidemiology in Taiwanese with asthma revealed that leukotriene receptor antagonists significantly decreased the risk of various cancers in a dose-dependent manner. In colorectal and breast cancers, the increased cysteinyl leukotriene receptor type 1 (CysLT1R) expression and decreased cysteinyl leukotriene receptor type 2 (CysLT2R) expression are associated with a poor prognosis and decreased survival of patients. The present study investigated the expression of 5-LOX, CysLT1R and CysLT2R in meningiomas. Meningioma and non-cancerous dura tissues were collected from Prasat Neurological Institute, Bangkok, Thailand. Gene expression of 5-LOX, CysLT1R and CysLT2R (n=15) were measured using real time RT-PCR. Protein expression of 5-LOX was measured using Western blotting. Data were presented as the median with interquartile range and analyzed by Wilcoxon signed rank test. Meningioma tissues showed significantly higher expression of 5-LOX ($p<0.01$) and CysLT1R ($p<0.01$) whereas the expression of CysLT2R ($p<0.05$) mRNA was significantly decreased in meningioma tissues compared to non-cancerous dura tissues. Further analysis of 5-LOX protein expression by Western blotting showed significant elevation of 5-LOX ($p<0.01$) in meningioma compared to non-cancerous dura tissues (n=8). These results indicated the alteration of leukotriene level and cysteinyl leukotriene receptors in meningioma pathogenesis. Therefore, the expression levels of 5-LOX, CysLT1R and CysLT2R could be potential biomarkers of prognosis for meningioma and the inhibition of leukotriene pathway could be a novel target for meningioma treatment.

Keywords: Leukotriene, lipoxygenase, cysteinyl leukotriene receptor, meningioma

การเพิ่มขึ้นของการแสดงออกของ 5-Lipoxygenase และตัวรับลิวโคไตรอีนชนิดที่ 1 และการลดลงของการแสดงออกของตัวรับลิวโคไตรอีนชนิดที่ 2 ในมะเร็งเยื่อหุ้มสมอง

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บทคัดย่อ

มะเร็งเยื่อหุ้มสมองพบมากเป็นอันดับสองของมะเร็งสมองทั้งหมด อีกทั้งผลการรักษามะเร็งชนิดนี้ยังไม่ได้ผลดีเท่าที่ควรและยังไม่มีวิธีรักษาที่เป็นมาตรฐานหรือตัวบ่งชี้ทางชีวภาพ ลิวโคไตรอีนเป็นสารอักเสบที่มีบทบาทสำคัญในพยาธิสรีรวิทยาของโรคหอบหืดและมะเร็งหลายชนิด รายงานการศึกษาในผู้ป่วยหอบหืดจากประเทศไต้หวันพบว่า ผู้ป่วยที่ใช้ยายับยั้งตัวรับลิวโคไตรอีนมีปัจจัยเสี่ยงที่ลดลงในการเกิดมะเร็ง การศึกษาในมะเร็งลำไส้ใหญ่และมะเร็งเต้านมพบว่า การเพิ่มขึ้นของการแสดงออกของตัวรับ cysteinyl leukotriene ชนิดที่ 1 (CysLT1R) และการลดลงของการแสดงออกของตัวรับ cysteinyl leukotriene ชนิดที่ 2 (CysLT2R) มีความเกี่ยวข้องกับการพยากรณ์โรคที่ไม่ดีและลดการรอดชีวิตของผู้ป่วย งานวิจัยนี้มุ่งศึกษาการแสดงออกของลิวโคไตรอีน (5-LOX), CysLT1R และ CysLT2R ในมะเร็งเยื่อหุ้มสมอง ซึ่งเนื้อเยื่อมะเร็งเยื่อหุ้มสมองและเนื้อเยื่อหุ้มสมองส่วนที่ไม่เป็นมะเร็งเก็บจากสถาบันประสาทวิทยา เพื่อนำไปศึกษาการแสดงออกของยีน 5-LOX, CysLT1R และ CysLT2R (n=15) ด้วยวิธี real time RT-PCR และวัดการแสดงของโปรตีน 5-LOX ด้วยวิธี Western blotting แล้วนำเสนอข้อมูลเป็นค่ามัธยฐานและค่าพิสัยควอไทล์ วิเคราะห์ข้อมูลทางสถิติด้วย Wilcoxon signed rank test พบว่ามะเร็งเยื่อหุ้มสมองมีการแสดงออกของยีน 5-LOX (p<0.01) และ CysLT1R (p<0.01) มากขึ้นอย่างมีนัยสำคัญทางสถิติ ส่วน CysLT2R (p<0.05) มีการแสดงออกลดลงกว่าเนื้อเยื่อหุ้มสมองส่วนที่ไม่เป็นมะเร็ง การศึกษาแสดงออกของโปรตีน 5-LOX ด้วยวิธี Western blotting พบว่า มะเร็งเยื่อหุ้มสมองมีการเพิ่มขึ้นของโปรตีน 5-LOX (p<0.01) มากกว่าเนื้อเยื่อหุ้มสมองส่วนที่ไม่เป็นมะเร็ง (n=8) การเปลี่ยนแปลงการแสดงออกนี้ชี้ให้เห็นความสำคัญของลิวโคไตรอีนและตัวรับในพยาธิกำเนิดของมะเร็งเยื่อหุ้มสมอง ซึ่งระดับการแสดงออกของ 5-LOX, CysLT1R และ CysLT2R อาจนำมาใช้เป็นตัวบ่งชี้ทางชีวภาพถึงการพยากรณ์โรคมะเร็งเยื่อหุ้มสมอง และการยับยั้งการแสดงออกของ CysLT1R อาจจะใช้เป็นเป้าหมายของการรักษามะเร็งเยื่อหุ้มสมองได้

คำสำคัญ: ลิวโครไตรอีน, ไลปอกซีจีเนส, ตัวรับลิวโคไตรอีน, มะเร็งเยื่อหุ้มสมอง

Introduction

The prevalence of meningioma, the most common form of tumors originated form meninges, was 4.14/100000 in Thai adult population in 2014 (1). Moreover, this value was gradually increased each year. Current chemotherapy drugs are not effective and there is no standard therapy for meningiomas. Therefore, identification of biomarkers involved in the development and progression of meningiomas will provide new therapeutic targets and effective treatment strategies that improve the survival time of patients.

Leukotrienes are proinflammatory lipid mediators that play an important role in the pathophysiology of asthma and other inflammation diseases such as allergic rhinitis (2) and cancers (3-5). A recent large epidemiology in Taiwanese with asthma revealed that leukotriene receptor antagonists (LTRAs) significantly decreased risk of lung, colorectal, gastric, liver and breast cancers in a dose-dependent manner (6). LTRA users showed 60-78% decrease of cancer risk compared to LTRA non-users in lung, colorectal, liver and breast cancers. However, due to a small number of patients the correlations between LTRAs and brain cancer remains unclear. Overexpression of 5-lipoxygenase (5-LOX) is detectable in the cytoplasm and nuclear envelops of brain tumor cells in meningioma and glioblastoma tissues using immunohistochemistry method (7). Cysteinyl leukotriene receptors type 1 and 2 (CysLT1R and CysLT2R) are overexpressed in human glioma cell and associated with glioma cell proliferation (7). Inhibition of CysLT1R decreases the migration and invasion of glioma cell lines (8). Herein, we investigated the expression of 5-LOX, CysLT1R, and CysLT2R in meningiomas.

Materials and Methods

Tissues collection

Meningioma and non-cancerous dura tissues were collected via surgery from patients (n=15) with the initial suspicion of meningiomas at Prasat Neurological Institute, Bangkok, Thailand. This research project was approved by the committee on human rights related to research involving human subjects of Prasat Neurological Institute. Project number is 58020. All tissues were stored at -80 °C until use.

RNA extraction and purification

Meningioma and non-cancerous dura tissues were homogenized in lysis buffer and RNA was extracted using extraction columns by Total RNA Purification kit according to manufacturer's protocol (Jena Bioscience, Germany). Then, RNA concentration was determined by Nanodrop® spectrophotometer 2000/2000c. After that, complementary DNA (cDNA) was synthesized using 1 µg of total RNA mixed with random hexamer primers (Roche Diagnostics, USA) and superscript III reverse transcriptase (Invitrogen, USA) at 55^{o}C for 1 hour.

Real-time reverse transcriptase polymerase chain reaction (real-time RT-PCR)

The mRNA expression of 5-LOX, CysLT1R, and CysLT2R were determined using the real-time RT-PCR method with SensiFAST SYBR LO-ROX (Bioline, Canada) detection on an Applied Biosystems real time PCR 7500 system. Gene expression levels were determined with specific primers as listed in Table 1. Expression of target genes were normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels. The relative fold change of gene expression was calculated using the $2^{-\Delta\Delta ct}$ method.

Table 1 Real-time RT-PCR primer sequences (8).

Gene	Primer sequences 5′ to 3′ orientation
5-LOX	Forward: 5′TACATCGAGTTCCCCTGCTAC3′ Reward: 5′GTTCTTTACGTCGGTGTTGCT3′
CysLT1R	Forward: 5′GTAGGCTTCTTTGGCAATGG3′ Reward: 5′AGCCAAATGCCTTTGTGAAC3′
CysLT2R	Forward: 5′TCCACTTGACGACATGGAAA3′ Reward: 5′GTTCTTTACGTCGGTGTTGCT3′
GAPDH	Forward: 5′AGCCTTCTCCATGGTGGTGAAGAC3′ Reward: 5′CGGAGTCAACGGATTTGGTCG3′

Western blotting

Proteins were extracted using lysis buffer [20 mM Tris, 1% NP-40, 50 mM NaCl and Protease Inhibitor Cocktail (PIC) Set III (2:1000, Calbiochem, La Jolla, CA)]. Total protein levels were evaluated by Bradford assay. Proteins were separated by molecular weight (MW) using 10% SDS-PAGE gel electrophoresis and then transferred to nitrocellulose membrane. Proteins were detected by anti-5-lipoxygenase (BD Transduction Laboratories, Cat No. 610694, USA), and anti-GAPDH (Cell Signaling, Cat. No. 5174, USA) antibodies and horse-radish peroxidase (HRP) conjugated secondary antibodies. Band density was visualized on films, using enhanced chemiluminescence substrate (Bio-Rad, Cat. No. 170–5060, USA) and quantified using Image J (National Institutes of Health).

Data analysis

Real time RT-PCR and Western blotting data were presented as the median with interquartile range and analyzed by Wilcoxon signed rank test. Statistically significance was considered when the *p* value less than 0.05 using GraphPad® Prism version 5.0 (San Diego, CA).

Results

Altered mRNA expressions of 5-LOX, CysLT1R and CysLT2R in meningioma tissues

Meningioma tissues showed significantly higher expression of 5-LOX [5.75 (2.49, 8.43) vs 0.73 (0.33, 2.01)] and CysLT1R [6.45 (4.83, 12.56) vs 0.40 (0.27, 2.84)] compared to non-cancerous dura tissues (Figure 1 and Figure 2). In contrast, meningioma tissues had significantly lower expression of CysLT2R [0.26 (0.10, 0.96) vs 0.65 (0.30, 1.79)] compared to non-cancerous dura tissues (Figure 3).

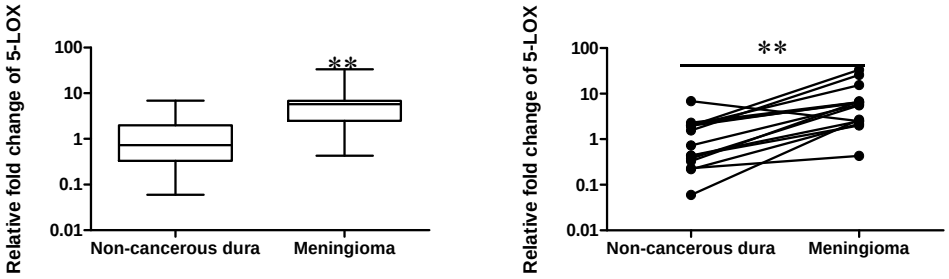

Figure 1. 5-LOX mRNA expression in meningioma and non-cancerous dura tissues from patients. The left graph showed median with interquartile range and the right graph showed match samples. n=15. ***p*<0.01, Wilcoxon signed rank test.

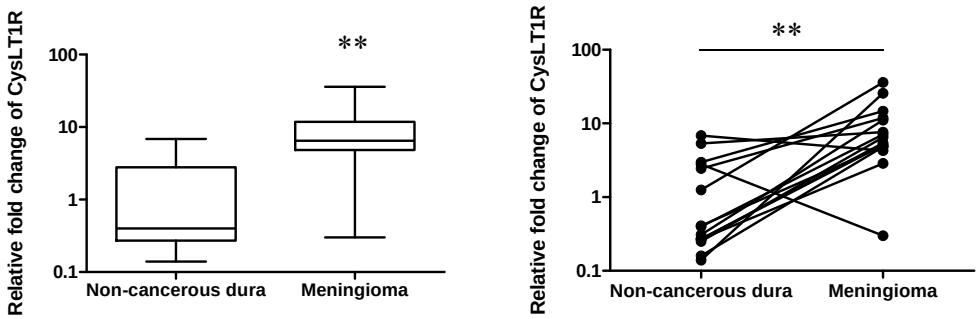

Figure 2. CysLT1R mRNA expression in meningioma and non-cancerous dura tissues from patients. The left graph showed median with interquartile range and the right graph showed match sample. n=15. ***p*<0.01, Wilcoxon signed rank test.

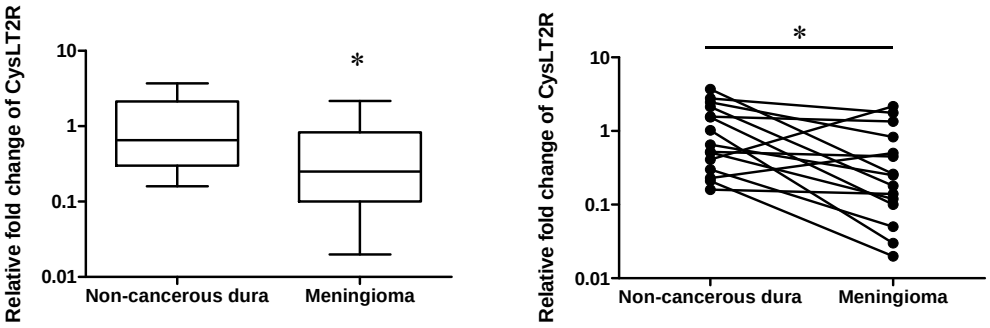

Figure 3. CysLT2R mRNA expression in meningioma and non-cancerous dura tissues from patients. The left graph showed median with interquartile range and the right graph showed match sample. n=15. **p*<0.05, Wilcoxon signed rank test**.**

Increased 5-LOX protein expression in meningioma tissues

A representative blot and bar graph showed significant elevation of 5-LOX [1.06(0.92, 1.31) vs 0.44(0.28, 0.63)] in meningioma compared to non-cancerous dura tissues (n=8) as shown in Figure 4.

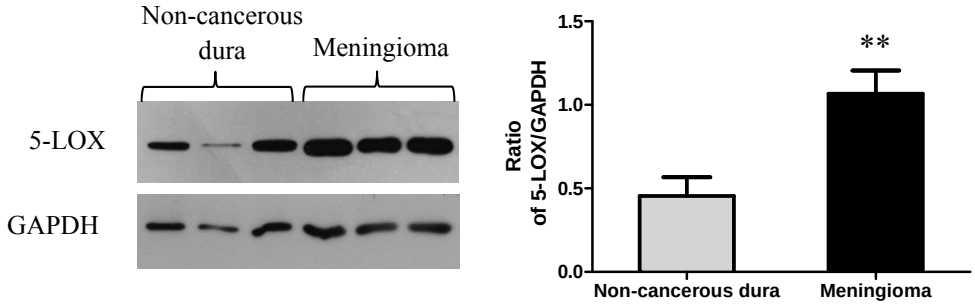

Figure 4. 5-LOX protein expression in meningioma (lane 4-6) and non-cancerous dura tissues (lane 1-3). The left of figure showed band density and the right graph showed ratio of 5-LOX/GAPDH. n=3. ***p*<0.01, Wilcoxon signed rank test.

Discussion

This study demonstrated the increased expressions of 5-LOX and CysLT1R together with the decrease expression of CysLT2R mRNA in meningioma tissues. 5-LOX and cysteinyl leukotriene receptors were expressed in various types of cancers including colon, esophagus, lung, renal, prostate, and breast cancers (9). A previous study in human brain tumors found 5-LOX mRNA expression in three meningioma, one astrocytoma and two glioblastoma tissues (10). Another immunohistological study showed the intensely staining of 5-LOX in the cytoplasm and nuclei in five meningioma, seven astrocytoma and six glioblastoma tissues (7). Consistently, the present study showed the elevation of 5-LOX mRNA and protein in meningioma compared to non-cancerous dura tissues. Our previous study reported that two cysteinyl leukotriene receptor antagonists, montelukast and zafirlukast, inhibited migration and invasion of A172 and U373 glioblastoma cells. Moreover, these drugs were also inhibited the matrix metalloproteinase 2/9 expression and activity, indicating that cysteinyl leukotriene plays a role in the migration and invasion of glioma (8). A study in 215 colorectal cancer patients suggested that the increased expression of CysLT1R and decreased expression of CysLT2R are associated with a poor prognosis in patients (11). In breast cancer, patients with high CysLT1R and low CysLT2R expressions (n=46) had significantly deceased survival time compared to patients with low CysLT1R and high CysLT2R expressions (n=71) (12). These studies indicate that pattern of CysLT receptor expression is associated with prognosis; therefore, further study in larger number of meningioma patients together with clinical outcome is required.

Conclusion

These results indicate the importance of 5-LOX and cysteinyl leukotriene receptors in meningioma pathogenesis. Therefore, levels of cysteinyl leukotrienes, synthesis enzyme and their receptors could be potential biomarkers of prognosis for meningioma and inhibition of 5-LOX and cysteinyl leukotriene receptors could be novel targets for meningioma treatment.

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B05 Effects of Caffeine on Ethanol-Induced Neurotoxicity in SH-SY5Y Differentiated Cells

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Abstract

Caffeine and ethanol are legal substances used for abuse; however, harmful when consumed together has been still unclear. This study aims to investigate whether caffeine potentiates the neurotoxicity of ethanol. The SH-SY5Y human neuroblastoma cells were cultured and induced with 10 μM retinoic acid for 3 days to allow for neuronal differentiation including an increase of tyrosine hydroxylase and dopamine transporter to use as the brain model. Thereafter, the cells were treated with caffeine, ethanol or a combination of ethanol and caffeine. The cell viability was assessed by an MTT assay and the nuclear morphology was visualized by the DNA staining with Hoechst 33258. Both ethanol and caffeine reduced cell viability in a concentration-dependent manner. Treatment with 400 mM ethanol for 24 hours significantly decreased the cell viability to 6.64 ± 0.99 % of control ($P < 0.0001$), and treatment with 20 mM caffeine for 24 hours significantly decreased the cell viability to 34.29 ± 2.84 % of control ($P < 0.0001$). With a combination experiment, the cell viability was decreased obviously. When visualized by the DNA staining, the cells showed an increase of condensed nuclei in a combination with caffeine after pretreated with ethanol. The study demonstrated that the effects of caffeine depend on the dose and also that combined pre-treatment of ethanol with caffeine at high doses significantly increased the toxic effects in differentiated SH-SY5Y cells. This suggests the potentiation effect of caffeine on ethanol neurotoxicity.

Keywords: caffeine, neurotoxicity, ethanol, SH-SY5Y cells, potentiating

ผลกระทบของคาแฟอีนต่อเอทานอลที่ทำให้เกิดความเป็นพิษต่อเซลล์ SH-SY5Y ที่เปลี่ยนเป็นเซลล์ประสาท

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บทคัดย่อ

คาแฟอีนและเอทานอลเป็นสารที่ใช้ในทางที่ผิดซึ่งนำมาใช้อย่างถูกกฎหมาย แต่อันตรายเมื่อบริโภคร่วมกันยังไม่ชัดเจน จึงมีจุดประสงค์เพื่อศึกษาหาว่าคาแฟอีนมีฤทธิ์ที่จะส่งเสริมฤทธิ์ของเอทานอลที่ทำให้เกิดพิษต่อสมองหรือไม่ โดยเพาะเลี้ยงเซลล์เนื้องอกในสมองชนิด SH-SY5Y แล้วชักนำด้วยกรดวิตามินเอความเข้มข้น 10 ไมโครโมลาร์ เป็นเวลา 3 วัน เพื่อให้เซลล์เปลี่ยนรูปร่างเป็นเซลล์ประสาท รวมถึงการเพิ่มขึ้นของเอ็นไซม์ไทโรซีนไฮดร็อกซิเลสและโดปามีนทรานสปอร์ทเตอร์เพื่อใช้เป็นแบบจำลองของสมอง หลังจากนั้นให้เซลล์สัมผัสกับเอทานอล, คาแฟอีน, หรือเอทานอลร่วมกันกับคาแฟอีนเทียบกับกลุ่มควบคุม ความสามารถในการรอดชีวิตของเซลล์ถูกประเมินด้วยวิธี MTT รวมถึงลักษณะของนิวเคลียสในเซลล์ด้วยสี Hoechst 33258 ที่ย้อมติดดีเอ็นเอ พบว่าความสามารถในการรอดชีวิตของเซลล์ลดลงขึ้นกับความเข้มข้น หลังจากที่ให้เซลล์สัมผัสเอทานอลความเข้มข้น 400 มิลลิโมลาร์ เป็นเวลา 24 ชั่วโมง เซลล์มีความสามารถในการรอดชีวิตลดลงอย่างมีนัยยะสำคัญอยู่ที่ 6.64 ± 0.99 % เมื่อเทียบกับกลุ่มควบคุม ($P < 0.0001$) ส่วนเซลล์ที่สัมผัสคาแฟอีนความเข้มข้น 20 มิลลิโมลาร์ เป็นเวลา 24 ชั่วโมง เซลล์มีความสามารถในการรอดชีวิตลดลงอย่างมีนัยยะสำคัญอยู่ที่ 34.29 ± 2.84 % เมื่อเทียบกับกลุ่มควบคุม ($P < 0.0001$) รวมถึงมีการลดลงของเซลล์ที่ยังมีชีวิตอย่างเห็นได้ชัดเมื่อย้อมด้วยสีย้อมติดดีเอ็นเอ มีการเพิ่มขึ้นของนิวเคลียสที่รวมตัวกันแน่นเมื่อเทียบกับกลุ่มควบคุม การศึกษานี้แสดงว่าผลกระทบจากคาแฟอีนนั้นขึ้นอยู่กับความเข้มข้น โดยเฉพาะเมื่อคาแฟอีนความเข้มข้นสูงแล้วมีการออกฤทธิ์ร่วมกันกับเอทานอล มีการเพิ่มขึ้นอย่างมีนัยยะสำคัญถึงความเป็นพิษต่อเซลล์ SH-SY5Y ที่ชักนำให้เป็นเซลล์ประสาท บ่งชี้ได้ว่าคาแฟอีนมีผลกระทบโดยการส่งเสริมฤทธิ์ของเอทานอลที่ทำให้เกิดความเป็นพิษต่อสมองได้

คำสำคัญ : คาแฟอีน , พิษต่อสมอง, เอทานอล, เซลล์ SH-SY5Y, ส่งเสริมฤทธิ์

Introduction

Caffeine is a substance commonly found in several beverages such as coffee, cola, and tea. It is a potent nervous system stimulant. Abuse results in symptoms of caffeinism which include agitation, disorientation and a syndrome which may be mistaken for anxiety/neurosis (1). Ethanol is a popular beverage of drug abuse worldwide including in Thailand. However, it acts as a teratogen by causing brain abnormalities (2) and enhancing cell death of differentiated neurons (3). Chronic effects of ethanol-induced oxygen radical formation and lipid peroxidation in rat brain (4), during ethanol catabolism, damaged mitochondria in responses to ethanol exposure may cause oxidative stress, resulting in neurodegeneration. Previous research showed that caffeine exerts its effects on the cardiovascular and neurological system, mainly through action as a non-selective adenosine antagonist (5), and processes activities on ryanodine receptor (6). In contrast, neuroprotective effects of caffeine have been reported through inhibition of adenosine A_{2A} receptor (7), and the pilot study reported that the combination of caffeine and ethanol provides robust neuroprotective in animal model (8) But in astrocytes, apoptotic effect of ethanol was potentiated by caffeine (9), and ethanol has been reported that it also has a synergistic effect with caffeine toxicity. Furthermore, it has been reported that caffeine can induce neuronal death in SH-SY5Y cells (10), which is the neuroblastoma cell line as an *in vitro* model of the dopaminergic neuron (11). Currently, many people increasingly consume both substances including mixed them together. For example it has been reported that 71% of young adults in Europe who consumed mixed caffeine and ethanol (12) Despite there are legal popular consumed in worldwide. But the harmful at high doses especially when consumed together, has been still controversial and unclear. Therefore, the question raised is whether or not caffeine can potentiate the effect on ethanol neurotoxicity in neurons via the apoptosis mechanism.

Material and Methods

Cell Culture

The SH-SY5Y human neuroblastoma cells were cultured in a 1:1 mixture of Modified Eagle Medium (MEM) and Nutrient Mixture Ham's F12 medium and supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1 mM sodium pyruvate, 0.1 mM nonessential amino acid, 1.5g/L sodium bicarbonate, 100 units/mL penicillin, and 100 µg/mL streptomycin. All media and supplements were purchased from Gibco (Gaithersburg, MD, USA). Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 incubator for 24 hours and treated with 10 µM retinoic acid for 3 days to allow for cells differentiation and increase tyrosine hydroxylase and dopamine transporter (13, 14). These cells expressed an increase of the tyrosine hydroxylase and the dopamine transporter and function like a mature neuronal phenotype used as an *in vitro* model for the neurotoxicity testing (16). In the experiment, cells were subcultured and plated into appropriate culture plates. The number of cells to be subcultured were assessed under a phase-contrast microscope based on the exclusion of trypan blue dye. The culture was maintained for 24 hours to allow for adhering to the plates. Thereafter, cells were treated with ethanol (Merck, Germany), caffeine (Sigma-Aldrich, St. Louis, MO, USA), or a combination of ethanol and caffeine according to the experiment design.

Measurement of Cell Viability

SH-SY5Y cells were seeded into a 96-well plate at a density of 1.0 x 10^4 cells/well in 100 µL of medium and incubated at 37°C under 5% CO_2 in a humidified incubator for 24 hours and

treated with 10 μM retinoic acid for 3 days to allow for cell differentiation and increase tyrosine immunoreactivity. After exposure to ethanol (50, 100, 200 and 400 mM) for 24 hour, caffeine (5, 10 and 20 mM) for 24 hour, or a combination of both by pre-treatment with ethanol for 24 hours and followed with caffeine for 24 hours, cell viability was measured by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay (Sigma-Aldrich, St. Louis, MO, USA). This method was based on the reduction of tetra ring of MTT by mitochondrial dehydrogenases with NADH in the active mitochondria, yielding a blue formazan product, which can be measured by a spectrophotometer (15). After incubation, 100 μL of MTT (5 mg/mL) was added to each well and the cell was cultured for another 3 hours, then the medium was removed, and 100 μL of DMSO (Sigma-Aldrich, St. Louis, MO, USA) was added to each well to dissolve the formazan. The color reaction was measured at wavelength 570 nm with a reference at 690 nm using the Bio-Tek microplate reader with 4 KC Synergy HT Software (Bio-Tek, China).

Staining of Nuclear DNA

SH-SY5Y cells were seeded on coverslips and fixed with 4% paraformaldehyde in 0.01 M PBS (pH 7.4) for 30 minutes at room temperature and then rinsed two times with PBS. After being washed with PBS, the cell was stained with 1 mg/mL Hoechst 33258 (Sigma-Aldrich, St. Louis, MO, USA) in PBS for 30 minutes at room temperature and then washed again. Coverslips were then mounted on the slide with anti-fade in mounting medium. Hoechst 33258 (bisbenzimide) preferentially binds to triplet adenine and thymine base pairs in the minor groove outside the double helix, which allows one to observe the morphological change in the nuclei of apoptotic cells (15). Nuclear morphology was examined under a fluorescent microscope (Nikon ECLIPSE TS-2000-S, Tokyo, Japan).

Statistical Analysis

All experiments were performed in triplicate (n = 3). Statistical analyses were performed with one-way ANOVA test followed by a post hoc analysis (Turkey's multiple comparison tests) using GraphPad Prism 5 Software for Windows (GraphPad Software, Inc., San Diego, CA, USA). All values were presented as the mean ± standard error of the mean (mean ± SEM) for each group. P <0.05 was considered statistically significant.

Results

Dose-dependent effects of caffeine and ethanol on cells death in differentiated SH-SY5Y cells

First, dose responses were investigated for both ethanol and caffeine at a 24 hour period on the cell viability of differentiated SH-SY5Y cells assessed by an MTT assay. Cell viability was decreased in a dose-dependent manner after treatment with ethanol or caffeine (Fig. 1a and Fig. 1b). After 24 hour treatment with 50 mM, 100 mM, 200 mM and 400 mM ethanol, cell viability was significantly decreased to 93.14 ± 4.94, 83.63 ± 5.73, 74.05 ± 5.12 and 6.64 ± 0.99 % of control respectively (P < 0.0001). For the concentrations of 5 mM, 10 mM and 20 mM for 24 hours, caffeine reduced cell viability to 92.55 ± 0.29, 78.99 ± 5.18 and 34.29 ± 2.84 respectively (P < 0.0001). Based on this data, we chose the ethanol concentration at 400 mM, which significantly reduced cells viability to <10 % of the control (6.64 ± 0.99*; P* < 0.0001), and the caffeine at 20 mM for 24 hours, which significantly reduced viability- about 60 % of the control (34.29 ± 2.84*; P* < 0.0001)- for the combination experiment to demonstrate whether there was the synergistic effect of caffeine on ethanol in differentiated SH-SY5Y cells.

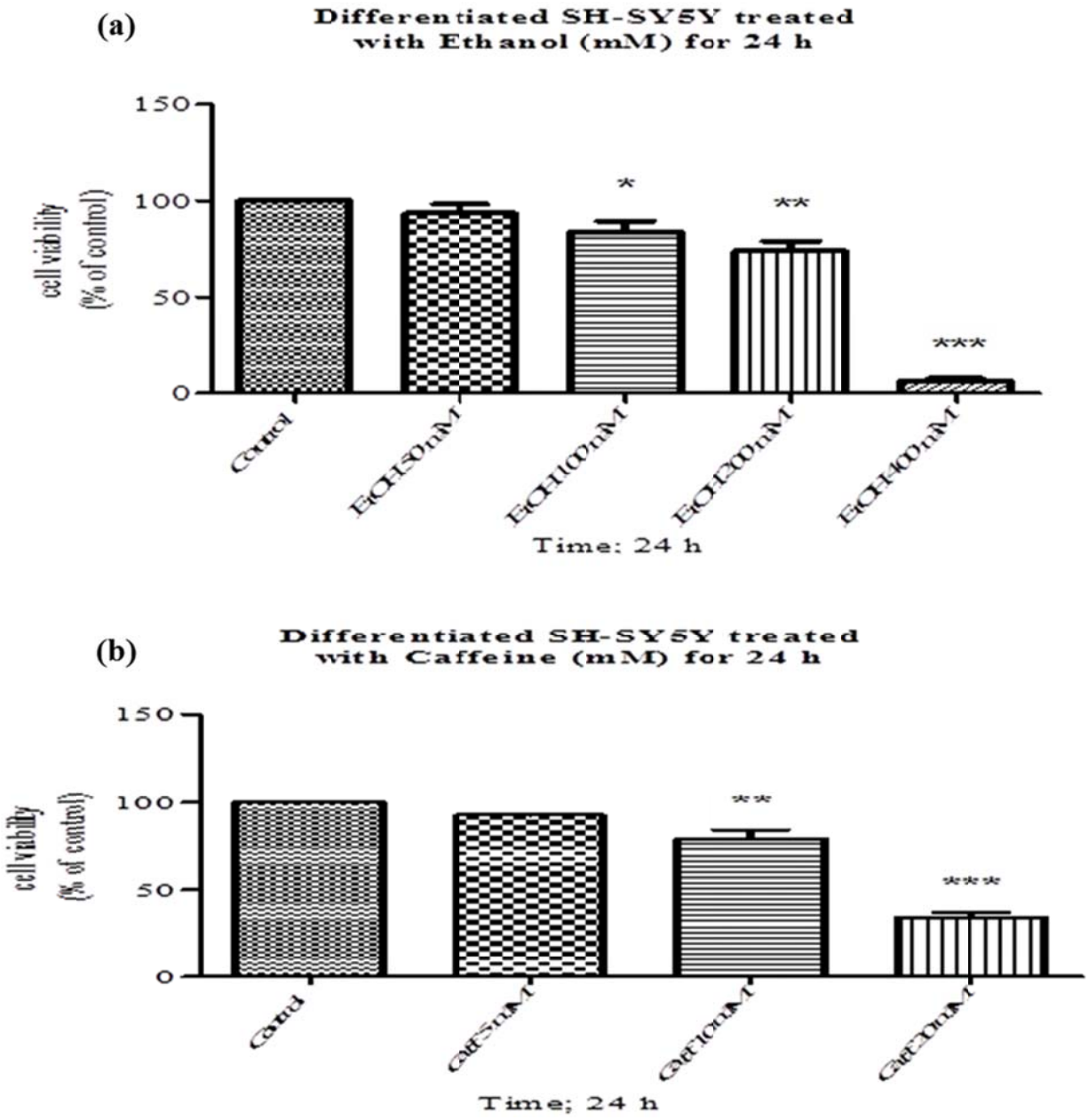

Figure 1. Ethanol or caffeine-induced cell death as assessed by an MTT assay. Cell viability of differentiated SH-SY5Y cells after treated with various concentrations of ethanol (a) or caffeine (b) for 24 hours, were reduced compared with untreated control as assessed by an MTT assay. Data are expressed as the mean of three replicate ± SEM of three independent experiments (*$P < 0.05$, **$P < 0.001$, ***$P < 0.0001$).

Caffeine increased cell death in ethanol-treated differentiated SH-SY5Y cells

Next, we performed the combination experiment with 400 mM ethanol treatment for 24 hours followed by 20 mM caffeine for 24 hours to examine cells death, assessed by an MTT assay. As shown in Fig.2 Hoechst 33258 staining was used to visualize nuclear fragmentation or condensation. Treatment with 400 mM ethanol followed by 20 mM caffeine increased the number of apoptotic nuclei in differentiated SH-SY5Y cells. When compared (b), (c) and (d) with the control reveals increasingly condensed nuclei in apoptotic cells especially a combination of caffeine after pretreated with ethanol for 24 hours (Fig. 2d).

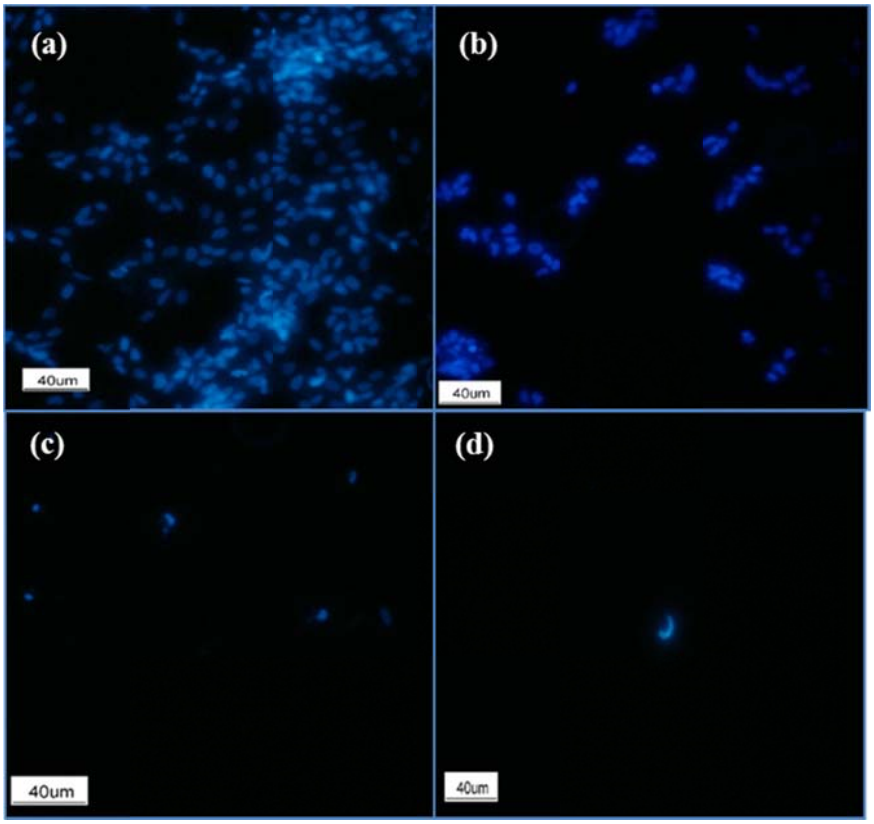

Figure 2. Effects of caffeine on nuclear morphology in ethanol pretreated in differentiated SHSY5Y cells. Control cells were untreated (a). Treated cells were exposed to 20 mM of caffeine (b), 400 mM of ethanol (c), or a combination of caffeine after pretreated with ethanol for 24 hours (d). Apoptotic nuclear morphology was visualized by DNA stain with Hoechst 33258. The results were performed three independent experiments.

Discussion

In this study, we investigated the synergistic effects of caffeine on ethanol toxicity in differentiated SH-SY5Y cells, which is the dopaminergic neuron that has a constant number because of a stopped proliferation. These cells express an increase of the tyrosine hydroxylase and the dopamine transporter and function like a mature neuronal phenotype used as an *in vitro* model for the neurotoxicity testing (16). Pre-treatment of ethanol and caffeine at high doses significantly decreased the cells viability of differentiated SH-SY5Y cells. Caffeine potentiated the number of cell deaths induced by ethanol, as demonstrated by an increase of condensed nuclei. Caffeine has been reported to have broad pharmacological activities such as neuroprotective against toxicity of MPTP and 6-hydroxydopamine in models of Parkinson'disease (17). Nevertheless, in this study we shown that both substances reduced cell viability in a concentration manner. Further, the combination study showed that caffeine potentiated the number of apoptotic cell deaths induced by ethanol as demonstrated by an increase of condensed nuclei in differentiated SH-SY5Y cells. This is in agreement with previous reports that the apoptotic effect of ethanol was potentiated by caffeine in astrocytes (9). From the data shown that apoptotic cells death in differentiated SH-SY5Y cells may cause of ethanol-induced reactive oxygen radicals and lipid peroxidation in the cell, including associated with oxidative stress due to damaged mitochondria after ethanol

exposure (4). Caffeine may exert its effects on the cell through inhibition of adenosine $A2_A$ receptor, which is a major target of caffeine, and induces ROS-mediated apoptosis through the mitochondria-mediated signaling pathway (6, 7, 18). Resulting in cellular stress and leading to apoptosis. Combined ethanol and caffeine showed increased apoptosis of cells compared to untreated control, ethanol alone and caffeine alone, due to ethanol was potentiated by caffeine (9). Although the doses used in this study are far from the doses daily consumed, the finding demonstrates that the effects of caffeine on ethanol-induced toxicity in neural cells may be a risk factor when consumed both substances at high doses together. Because of the PI3K/Akt/mTOR pathway plays a central role in cell growth, protein translation, survival, and metabolism (19). Importantly, the mTOR pathway senses and integrates a variety of environmental cues to regulate organismal growth and homeostasis. This pathway regulates many major cellular processes and was implicated in an increasing number of pathological conditions, including cancer, obesity, type 2 diabetes, and neurodegeneration (20). Therefore, further study on potentiating the effect of caffeine on ethanol neurotoxicity on this pathway should be performed.

Conclusion

The study demonstrated that the effects of caffeine depending on the doses and the combined treatment of ethanol and caffeine at high doses significantly increases the toxic effects in differentiated SH-SY5Y cells, suggesting the potentiation effect of caffeine on ethanol neurotoxicity. Thus, our findings provide preliminary evidence that high consumption of caffeine together with ethanol at high doses may be a risk factor for neurodegeneration in human. For the molecular pathways, which may influence such as the mechanistic target of rapamycin or mTOR signaling pathway should be considered for further study.

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B06 Anticancer Effects of Mansonone G derivatives on Human Colorectal Cancer Cells

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Abstract

Mansonone G (MG), a 1,2-naphthoquinone, isolated from heartwood of *Mansonia gagei* Drumm (Chan-Cha-Mod), exhibited several pharmacological activities, including anti-bacterial, anti-fungal, anti-estrogenic, anti-adipogenic and anti-cancer. However, the cytotoxic effects of mansonone G and its derivatives on colorectal cancer cell lines have not been investigated. Therefore, the objective of this study was to evaluate cytotoxic effects of MG and its derivatives on two colorectal cancer cell lines, HCT-116 and HT-29. MTT assay indicated that MG and its derivatives inhibited cell proliferation in a concentration-dependent manner in both colorectal cancer cell lines. IC_{50} values of MG and its derivatives, including G01, G02, G03, G04, G06, G07, G08, G09 and G10 were 20.74±0.51, 20.86±3.10, 10.60±0.65, 6.63±0.60, 2.77±0.20, 26.59±2.39, 6.13±0.59, 6.54±0.47, 10.83±1.44, 37.85±1.42, 5.16±0.53 µM, respectively, in HCT-116 cells and were 25.55±2.08, 15.07±1.91, 8.43±0.81, 8.09±1.13, 3.24±0.21, 26.68±2.08, 13.62±1.42, 5.66±1.13, 7.53±1.20, 98.59±3.37, 9.89±1.49 µM, respectively, in HT-29 cells. Remarkably, MG derivatives, including G01, G02, G03, G04, G06, G07, G08 and G10 were more toxic to colorectal cancer cells than the parent compound, especially G04 and G07. We however found that G07 was more toxic to cancer cells than normal human colon cells, CRL-1790. Additionally, flow cytometry analysis indicated that G07 was able to induce apoptotic cell death in both HCT-116 and HT-29 cells. These results suggested that MG derivatives may be a novel anticancer agent of colorectal cancer.

Keywords: Mansonone G, *Mansonia gagei* Drumm, colorectal cancer, cytotoxicity

ฤทธิ์ต้านมะเร็งของสารอนุพันธ์ของแมนโซโนนจีต่อเซลล์มะเร็งลำไส้ใหญ่และไส้ตรงของมนุษย์

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บทคัดย่อ

Mansonone G (MG) เป็นสารในกลุ่ม 1,2-naphthoquinone ที่สกัดได้จากเนื้อไม้ของต้น *Mansonia gagei* Drumm (ต้นจันทน์ชะมด) มีฤทธิ์ทางเภสัชวิทยาที่หลากหลาย ประกอบด้วย ฤทธิ์ต้านแบคทีเรีย ฤทธิ์ต้านเชื้อรา ฤทธิ์ต้านฮอร์โมนเอสโตรเจน ฤทธิ์ต้านเซลล์ไขมัน และฤทธิ์ต้านมะเร็ง แต่ยังไม่เคยมีรายงานเกี่ยวกับฤทธิ์ต้านมะเร็งของสาร MG และสารอนุพันธ์ของ MG ดังนั้นงานวิจัยนี้จึงมีวัตถุประสงค์ เพื่อศึกษาความเป็นพิษของสาร MG และสารอนุพันธ์ของ MG ทั้งหมด 10 ชนิดต่อเซลล์มะเร็งลำไส้ใหญ่และไส้ตรงของมนุษย์สองชนิด ได้แก่ HCT-116 และ HT-29 จากผลการศึกษาความเป็นพิษด้วยวิธี MTT พบว่า สาร MG และสารอนุพันธ์ของ MG สามารถยับยั้งการเจริญเติบโตของเซลล์มะเร็งแบบขึ้นอยู่กับความเข้มข้นทั้งในเซลล์ HCT-116 และ HT-29 โดยมีค่า IC_{50} ของสาร MG และสารอนุพันธ์ของ MG ได้แก่ G01, G02, G03, G04, G05, G06, G07, G08, G09 และ G10 มีค่าเท่ากับ 20.74±0.51, 20.86±3.10, 10.60±0.65, 6.63±0.60, 2.77±0.20, 26.59±2.39, 6.13±0.59, 6.54±0.47, 10.83±1.44, 37.85±1.42, 5.16±0.53 μM ตามลำดับในเซลล์ HCT-116 และมีค่าเท่ากับ 25.55±2.08, 15.07±1.91, 8.43±0.81, 8.09±1.13, 3.24±0.21, 26.68±2.08, 13.62±1.42, 5.66±1.13, 7.53±1.20, 98.59±3.37, 9.89±1.49 μM ตามลำดับในเซลล์ HT-29 และพบว่าสารอนุพันธ์ MG หลายชนิด ได้แก่ G01, G02, G03, G04, G06, G07, G08 และ G10 สามารถยับยั้งการเจริญเติบโตของเซลล์ได้ดีกว่าสาร MG โดยเฉพาะสารอนุพันธ์ G04 และ G07 อย่างไรก็ตามเราพบว่าสาร G07 มีความเป็นพิษต่อเซลล์มะเร็งมากกว่าเซลล์ลำไส้ปกติ คือ CRL-1790 และจากการวิเคราะห์ด้วยเทคนิค flow cytometry พบว่าสาร G07 สามารถเหนี่ยวนำให้เซลล์ HCT-116 และ HT-29 เกิดการตายแบบอะพอพโตซิส การทดลองนี้แสดงให้เห็นว่าสารอนุพันธ์ของ MG อาจนำมาพัฒนาใช้เป็นยาต้านมะเร็งลำไส้ใหญ่และไส้ตรงต่อไปได้

คำสำคัญ: แมนโซโนนจี, จันทน์ชะมด, มะเร็งลำไส้ใหญ่และไส้ตรง, ความเป็นพิษต่อเซลล์

Introduction

Colorectal cancer (CRC) is one of the most common cancer among men and women (1). In 2016, the American Cancer Society reported that colorectal cancer is one of the most common human malignant diseases and the third leading cause of cancer-related death throughout the world (2). Treatment options for colorectal cancer include surgery, radiotherapy, chemotherapy and targeted therapy (3). Although chemotherapy has been widely used, severe side effects and acquired drug resistance have limited its application. Therefore, new compounds with potent anticancer activity are urgently needed.

Mansonones, including mansonone C, E, F, G, H, I, N, O, P and S, isolated from *Mansonia gagei* Drumm, have shown several pharmacological activities such as anti-bacterial, anti-fungal, anti-estrogenic, anti-adipogenic and anti-cancer (5-8). It was reported that mansonone E and mansonone F could inhibit proliferation of many cancer cell types including breast cancer, colon cancer, melanoma, cervical cancer, lymphoma and oral cancer (9-10). Previous study reported that structurally modification on ether analogues of mansonone G (MG), a major compound isolated from *Mansonia gagei* Drumm, made the compounds more hydrophobic, which may facilitate the access to bacterial cell wall, resulting in higher antibacterial activity (5). However, the cytotoxicity of MG and its derivatives against colorectal cancer cells has not been reported. In this study, we evaluated the cytotoxic effects of MG and its derivatives on two human colorectal cancer cell lines, HT-29 and HCT-116.

Materials and Methods

Cell culture

The human colon carcinoma cell lines, HCT-116 and HT-29, were obtained from the American Type Culture Collection (ATCC). HCT-116 cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin. HT-29 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin. Cells were grown in a humidified 5% CO_2 incubator at 37°C.

Cytotoxicity assay

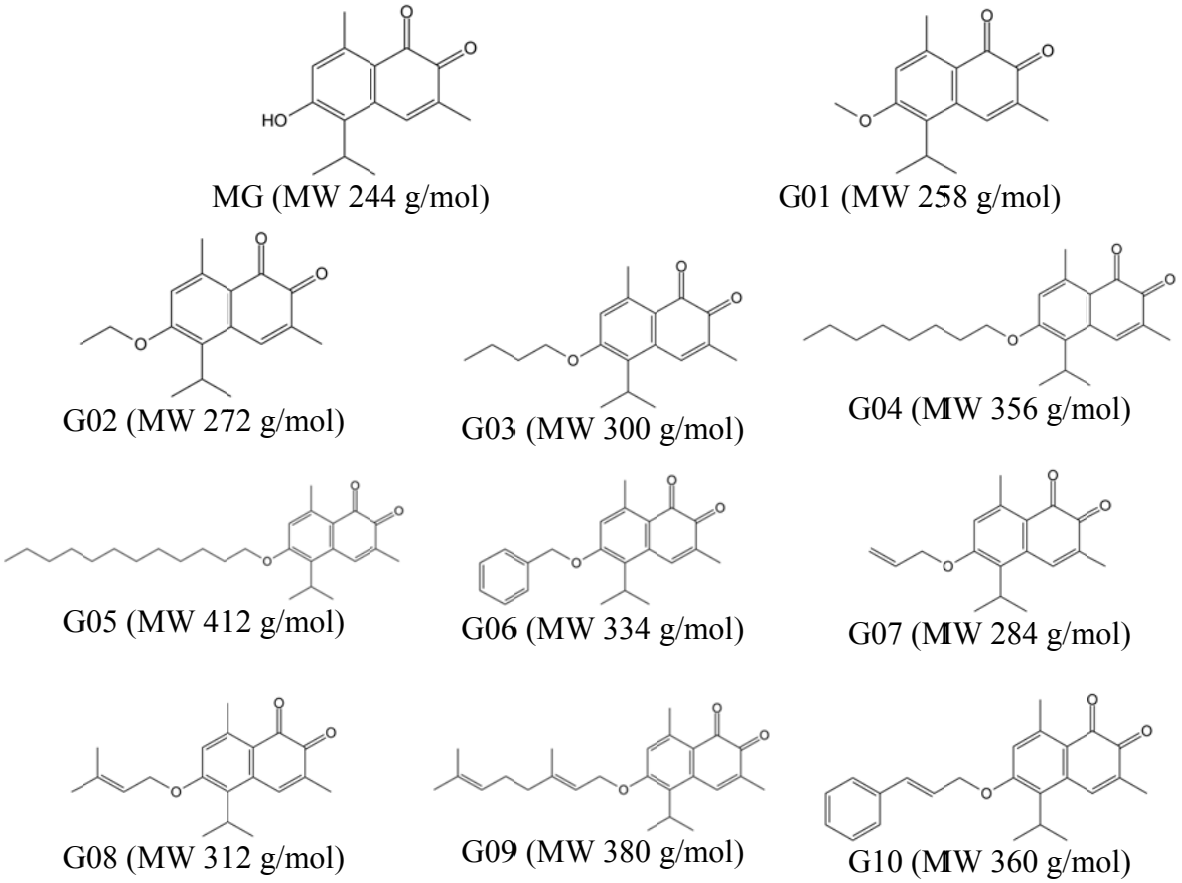
The cytotoxic effects of MG and its derivatives were determined by MTT assay. Briefly, cells were seeded in 96-well plates at a density of $5x10^4$ cell/ml and incubated overnight at 37 °C. The cells were then treated with MG or its derivatives, including G01, G02, G03, G04, G05, G06, G07, G08, G09 and G10, or 0.2% DMSO (vehicle control) in complete medium at the concentrations of 0.1, 1, 10 or 100 µM for 48 h. Next, 15 µL of MTT (0.5 mg/mL) was added into each well and incubated the cells for 4 h. After removing supernatant, 150 µL of DMSO was added to solubilize formazan crystals. We measured optical density at 570 nm using a microplate reader (Thermo, Finland).

Apoptosis assay

HCT-116 and HT-29 cells were seeded into 6-well plates at a density of $5x10^4$ cell/ml and incubated overnight. HCT-116 cells were then treated with G07 at concentrations of 2.5, 5 and 10 µM for 24 h. HT-29 cells were treated with G07 at concentrations of 5, 10 and 20 µM for 24 h. After that, the cells were collected by trypsinization, washed twice with PBS and stained with Annexin V FIT-C (Invitrogen, USA) and PI (Santa Cruz Biotechnology,

USA) for 15 min in the dark. The stained cells were analyzed using flow cytometer (BD LSR II, Biosciences).

Structures of MG and its derivatives



Statistical analysis

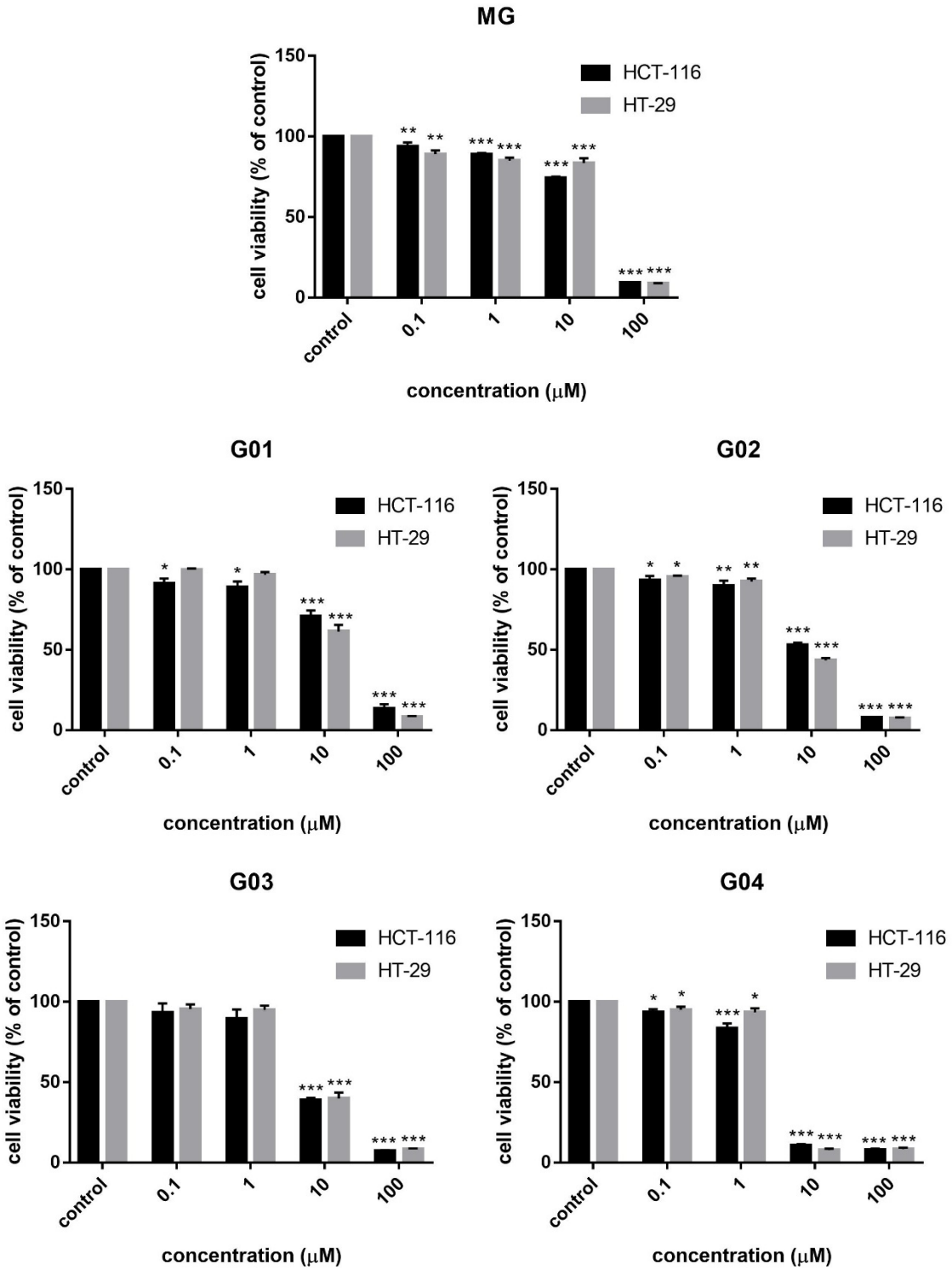
All data are reported as mean ± standard error of mean (SEM) from three independent experiments performed in triplicate. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by LSD using SPSS software program. Difference is considered significant if P value < 0.05.

Results

Effect of MG and its derivatives on cytotoxicity and apoptosis induction of HCT-116 cells and HT-29 cells

As shown in Fig.1, MG and its derivatives significantly inhibited the growth of both human colorectal cancer cell lines, HCT-116 and HT-29, in a concentration-dependent manner ($P<0.05$). It however should be noted that several MG derivatives, including G01, G02, G03, G04, G06, G07, G08 and G10 were more cytotoxic to cancer cells than the parent compound. Of all 11 compounds, G04 displayed the most potent cytotoxicity against HCT-116 cells, followed by G10, G06 and G07, respectively (Table 1). Similarly, G04, G07, G08 and G03 showed very potent cytotoxicity against HT-29 cells (Table 1). Although G04 and G07 were highly effective in controlling the growth of colorectal cancer cells, we found that G04 was more toxic to normal colon CRL-1790 cells than G07 (data not shown). Given its higher cytotoxicity against cancer cells than normal cells, we then evaluated apoptosis-

inducing effect of G07. Flow cytometry analysis indicated that G07 could induce cells to undergo apoptosis in HCT-116 and HT-29 cells (Figure 2). However, it should be noted that G07 induced cells to undergo late apoptosis rather than early apoptosis. Treatment of G07 at 2.5, 5 and 10 μM could induce HCT-116 cells to undergo late apoptotic about 3.31±1.60, 6.00±2.02 and 51.51±5.99%, respectively. Similarly, the percentage of late apoptotic HT-29 cells were about 4.82±1.98%, 5.68±2.02% and 74.63±9.95% following treatment with G07 at 5, 10 and 20 μM, respectively. These results suggested that G07 induced cell death via apoptosis induction.



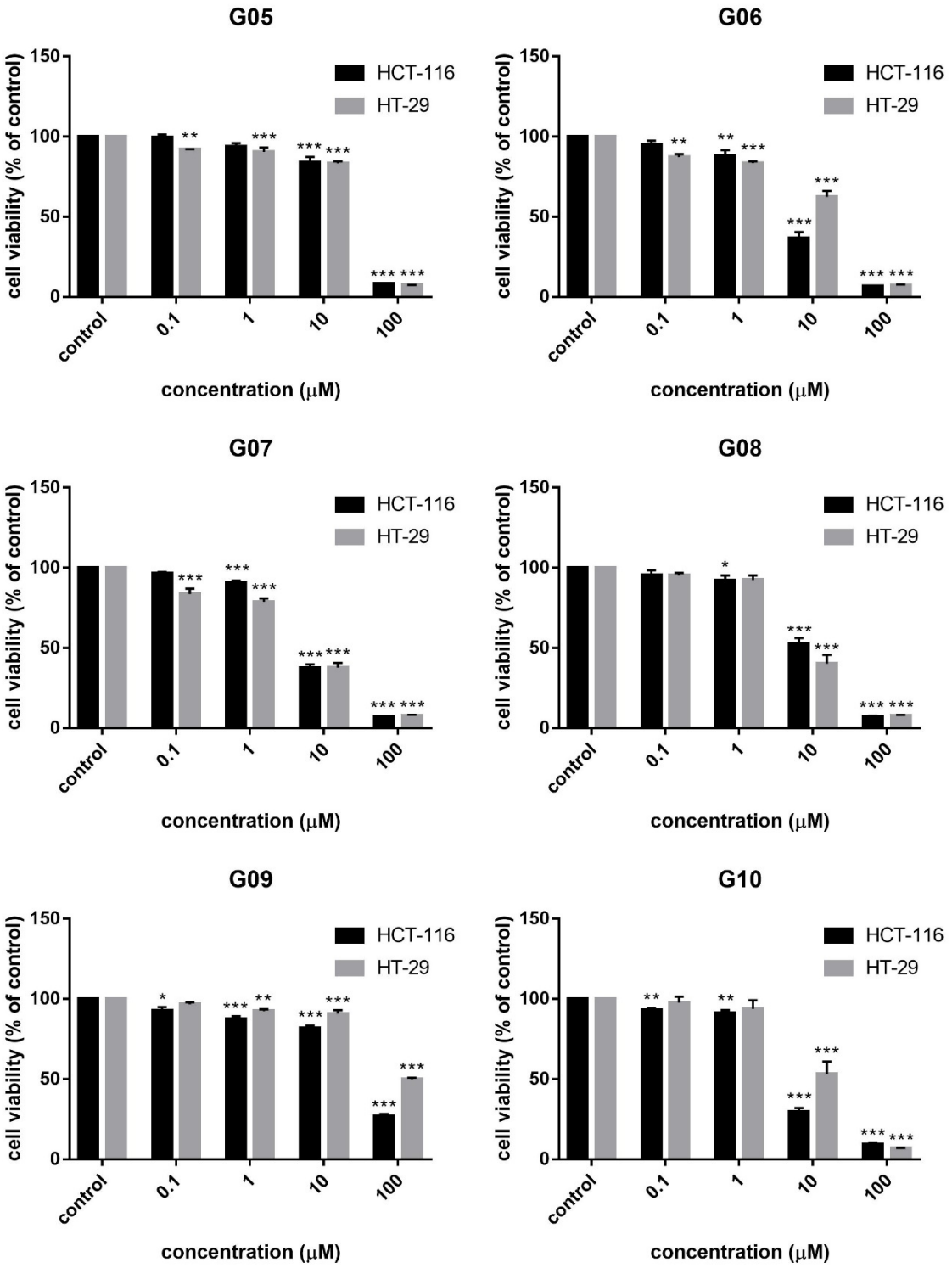


Figure 1. Effects of MG and its derivatives on cell viability of HCT-116 and HT-29 cells. Both cell lines were treated with MG or its derivatives at 0.1, 1, 10, 100 μM for 48 h. Cell viability was evaluated using MTT assay. Each value is expressed as the mean ± SEM (n=3). $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ compared with vehicle control (0.2%DMSO).

Table 1. IC_{50} values of MG and its derivatives on HCT-116 and HT-29 cells

Compounds	IC_{50} (μM)	
	HCT-116	HT-29
MG	20.74 ± 0.51	25.55 ± 2.08
G01	20.86 ± 3.10	15.07 ± 1.91
G02	10.60 ± 0.65	8.43 ± 0.81
G03	6.63 ± 0.60	8.09 ± 1.13
G04	2.77 ± 0.20	3.24 ± 0.21
G05	26.59 ± 2.39	26.68 ± 2.08
G06	6.13 ± 0.59	13.62 ± 1.42
G07	6.54 ± 0.47	5.66 ± 1.13
G08	10.83 ± 1.44	7.53 ± 1.20
G09	37.85 ± 1.42	98.59 ± 3.37
G10	5.16 ± 0.53	9.89 ± 1.49

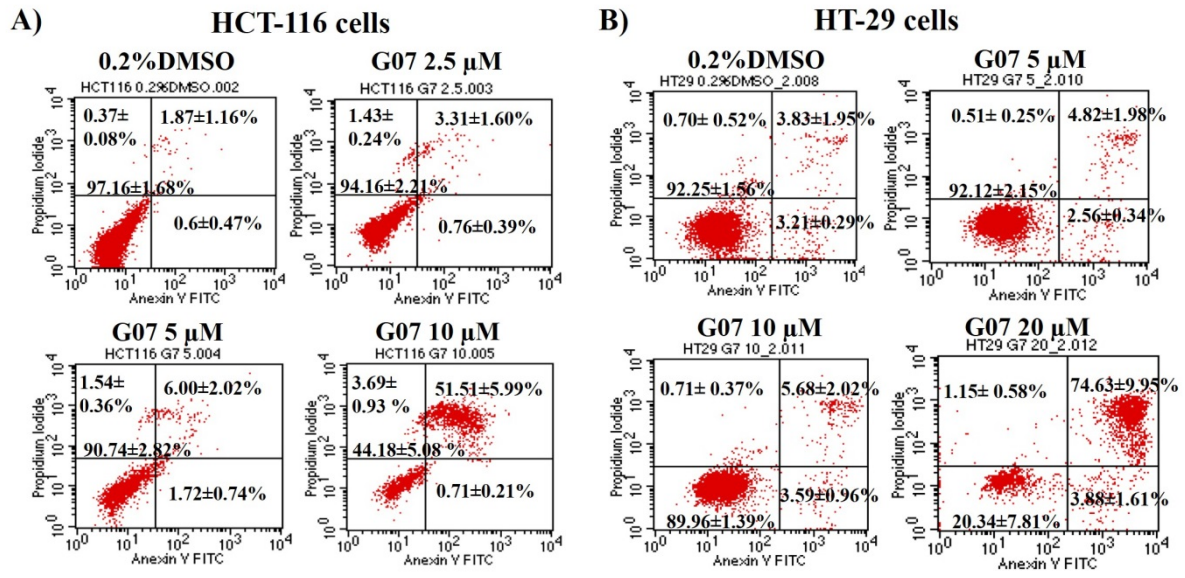


Figure 2. Apoptosis-inducing effect of G07 on colorectal cancer cells. A) Representative cytograms of cell apoptosis analysis of HCT-116 cells after treatment with G07 (2.5, 5, 10 μM) for 24 h. B) Representative cytograms of cell apoptosis analysis of HT-29 cells after treatment with G07 (5, 10, 20 μM) for 24 h. Each value is expressed as the mean ± SEM (n=3).

Discussion

Previous studies reported that mansonones, 1,2-naphthoquinones, isolated from heartwood of *Mansonia gagei* Drumm, exhibited several pharmacological activities such as anti-bacterial, anti-fungal, anti-estrogenic, anti-adipogenic and anti-cancer (5-8). The present study found that mansonone G (MG) exhibited cytotoxicity against colorectal cancer cells.

Notably, several ether analogs of MG displayed higher cytotoxicity than the parent compound. Previously, it was reported that structurally modification to ether analogs of MG resulted in improved antibacterial activities through facilitating the access to the lipophilic cell wall bacterial (5). Thus, it is likely that increased uptake of ether analogs of MG into cancer cells led to higher cytotoxicity when compared with the parent one. Additionally, we found that several compounds such as G09 and G10 were more toxic toward p53 wild-type HCT-116 cells than p53 mutant HT-29 cells. Since p53 mutations are commonly associated with resistance to many chemotherapeutic agents (11), it is possible that p53 plays a significant role on cytotoxicity of G09 and G10. It however should be noted that, of all 11 compounds, G04 and G07 displayed potent cytotoxicity against both p53 wild-type HCT-116 and p53 mutant HT-29 cells, suggesting that cytotoxicity of these two compounds is not primarily mediated through p53. It has been shown that naphthoquinone exert their anti-cancer activity via reactive oxygen species (ROS) generation. Previous study reported that 1,4-napthoquinone derivatives induce apoptosis through production of reactive oxygen species (ROS) on HCT-116 colon cancer cells (12). Therefore, cytotoxicity of G04 and G07 may be associated with ROS production, which requires further elucidation.

Conclusion

The present study clearly demonstrated that several ether analogs of MGs, including G01, G02, G03, G04, G05, G06, G07, G08, G09 and G10 were possesses high cytotoxicity on human colorectal cancer cell lines, HCT-116 and HT-29. Of all potent compounds, G07 was more cytotoxic activity toward cancer cells than normal cells. Apoptosis-inducing effects of G07 were also detected in colorectal cancer cells. These finding suggested that G07 is a promising anticancer agent for colorectal cancer; however, further investigation is needed to reveal the underlying mechanisms.

Acknowledgements

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B07 Photoprotective Effects of Oxyresveratrol Isolated from Heartwood of *Artocarpus lakoocha* in UVB Irradiated Human Keratinocyte, HaCaT cells

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Abstract

Background: Ultraviolet (UV) radiation from sunlight, especially UVB (280-320 nm) is a main cause of oxidative skin damage which can lead to serious skin problems including, edema, sunburn, and skin cancers. Over the years, oxyresveratrol has been intensively studied for the whitening effect, but its anti-oxidative activity on UVB-irradiated HaCaT cells has rarely been elucidated.
Objective: The aim of this study was to investigate the effects of oxyresveratrol on UVB-irradiated HaCaT cells.
Materials and Methods: The cell protective effects of oxyresveratrol on HaCaT cells after UVB radiation exposure were examined by resazurin assay for cell viability detection. In addition, the effects of oxyresveratrol on nitric oxide (NO) and reactive oxygen species (ROS) productions were evaluated by Griess assay and ROS assay, respectively.
Results: UVB-irradiated HaCaT cells significantly reduced the cell survival and increased in NO and ROS levels. In contrast, pretreatment of HaCaT cells with oxyresveratrol before exposure to UVB radiation could improve the reduction of cell viability and also reduced the productions of NO and ROS.
Conclusion: These finding together indicated that oxyresveratrol has an antioxidant role leading to indirectly protect the HaCaT cells from UVB radiation damage. Therefore, oxyresveratrol can be a potential compound for prevention of UVB-induced skin damage.

Keywords: Oxyresveratrol, UVB, HaCaT cells, anti-oxidant

ฤทธิ์ป้องกันอันตรายจากแสงของสาร oxyresveratrol ที่แยกได้จากแก่นของ *Artocarpus lakoocha* ต่อเซลล์เคอราติโนไซต์ HaCaT ที่ถูกฉายด้วยรังสียูวีบี

บทคัดย่อ

ความเป็นมา: รังสียูวีจากแสงแดด โดยเฉพาะอย่างยิ่งรังสียูวีบี (280-320 นาโนเมตร) เป็นสาเหตุหลักที่ทำให้ผิวหนังได้รับความเสียหายเนื่องจากภาวะออกซิเดชั่น ซึ่งสามารถนำไปสู่ภาวะผิดปกติของผิวหนังที่มีความร้ายแรง เช่น บวม ผิวหนังไหม้แดดและมะเร็งผิวหนัง ในช่วงหลายปีที่ผ่านมา ได้มีการศึกษาฤทธิ์ของสาร oxyresveratrol ที่มีผลทำให้ผิวหนังขาวกันอย่างกว้างขวาง แต่ฤทธิ์ต้านอนุมูลอิสระของสาร oxyresveratrol นั้น ยังไม่มีการศึกษามากนัก
วัตถุประสงค์: งานวิจัยครั้งนี้มีวัตถุประสงค์เพื่อศึกษาถึงผลของสาร oxyresveratrol ต่อเซลล์เคอราติโนไซต์, HaCaT ที่ถูกฉายด้วยรังสียูวีบี
วัสดุและวิธีการทดลอง: การศึกษาฤทธิ์ของสาร oxyresveratrol ต่อเซลล์ HaCaT หลังจากฉายรังสียูวีบี ศึกษาโดยวิธี resazurin assay ซึ่งใช้เพื่อวัดอัตราการรอดชีวิต นอกจากนี้ ผลของสาร oxyresveratrol ต่อปริมาณอนุมูลอิสระ nitric oxide (NO) และ reactive oxygen species (ROS) ศึกษา โดยวิธี Griess assay และ ROS assay ตามลำดับ
ผลการทดลอง: เซลล์ HaCaT ที่ถูกฉายด้วยรังสียูวีบี มีอัตราการรอดชีวิตของเซลล์ลดลงอย่างมีนัยสำคัญทางสถิติ และรังสียูวีบียังกระตุ้นให้เซลล์สร้าง NO และ ROS ในปริมาณที่สูงขึ้น การใส่สาร oxyresveratrol ไปที่เซลล์ HaCaT ก่อนการสัมผัสกับรังสียูวีบี สามารถเพิ่มอัตราการรอดชีวิตของเซลล์อย่างมีนัยสำคัญทางสถิติและยังสามารถลดปริมาณ NO และ ROS ได้อีกด้วย
สรุป: จากผลการทดลองที่ได้บ่งชี้ว่า สาร oxyresveratrol มีฤทธิ์โดยตรงในการลดสารอนุมูลอิสระ ซึ่งนำไปสู่ผลโดยอ้อมต่อการปกป้องเซลล์ HaCaT จากความเสียหายที่เกิดจากการได้รับรังสียูวีบี ดังนั้น สาร oxyresveratrol เป็นสารที่มีศักยภาพสำหรับการป้องกันความเสียหายของผิวหนังที่เกิดจากการได้รับรังสียูวีบีได้

คำสำคัญ: สาร oxyresveratrol รังสียูวีบี เซลล์ HaCaT ฤทธิ์ต้านอนุมูลอิสระ

Introduction

Nowadays, the amount of ultraviolet B (UVB) radiation (280-320 nm) reaching to the Earth's surface is increasing in every year due to the global warming and ozone layer depletion (1). Without the protection of ozone layer, the excessive amounts of UVB radiation can cause many harmful impacts to human e.g. genotoxic effect (2), photoproducts, ROS generation, and oxidative damage, plants and marine lives, and other environments (3). Although the skin has several defensive mechanisms to avoid skin damage, excessive UVB exposure can lead to serious skin problems such as hyperpigmentation, erythema, edema, sunburn, and melanoma and non-melanoma skin cancers (4).

Since the natural products is generally considered to be safe and have undesired harmful effect. The study of the natural bioactive compound modulating UVB-induced skin damage has been growing interest. Oxyresveratrol (trans-2,3´,4,5´- -tetrahydroxystilbene) is a natural hydroxystilbene. It is abundantly found in the heartwood of *Artocarpus lakoocha* which known as Ma-Haad in Thai. This plant widely distributed in the regions of South and South-East Asia (5), including Thailand. Oxyresveratrol shows various pharmacological effects such as anti-allergic effect (6), neuroprotective effects (7), tyrosinase inhibitory activity (8), and antioxidative effects (9). In this study, we investigated the antioxidant and protective effects of oxyresveratrol on UVB-induced skin photodamage by using keratinocytes, HaCaT cells as a model.

Materials and Methods

Materials

Keratinocytes, HaCaT cells were purchased from Cell Line Service, (Eppelheim, Germany). Dulbecco Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), and penicillin and streptomycin, phosphate-buffered saline (PBS) were purchased from Gibco BRL (Grand Island, NY, USA), Resazurin assay kit from Sigma (St. Louis, MO, USA) and Griess assay kit from Promega (Madison, WI, USA).

Oxyresveratrol

Oxyresveratrol used in this study was kindly provided by Assoc. Prof. Dr. Sunit Suksamran, Department of Chemistry, Faculty of Science, Srinakharinwirot University, Bangkok, Thailand.

Cell cultures

HaCaT cells were maintained in DMEM supplemented with 10% heat-inactivated FBS and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) in a 37°C, 5% CO_2, 95% humidified incubator. Oxyresveratrol was dissolved in dimethyl sulfoxide (DMSO) as a 200 mM stock solution before dilution to 0.1% of DMSO in each concentration to use in our experiment.

UVB irradiation

The UVB source (280-320 nm) was provided by parallel bank of 5x8-watt tubes of UVB Bio-Link BLK Cross linker (BIO-LINK). The UV intensity was measured using a VLX-3W radiometer (Vilber Lourmat, France). Prior to UVB irradiation, media was removed from the cells, and then the cells were washed with phosphate-buffer saline (PBS), and covered with thin layer of PBS followed by irradiating under UVB 40 mJ/cm^2 and placing on ice cold pack.

Resazurin assay

This cell viability assay was based on the ability of mitochondrial reductase for reducing resazurin (nonfluorescent, deep blue) into the resorufin, a magenta-pink and fluorescent dye (reduced form). Briefly, HaCaT cells cultured in 96-well plates ($4x10^4$cells/well) were treated with various concentrations of oxyresveratrol and further incubated for 24 h. In addition, the cells cultured in 35 mm cell culture dish ($3x10^5$ cells/dish) were pretreated with oxyresveratrol for 1 h, followed by UVB irradiation (40 mJ/cm^2) and then incubated for 24 h. After the treatment, the cells were added resazurin solution (10% of the culture media volume), incubated in a 37°C, 5% CO_2 humidified incubator in the dark for 2 h, and immediately measured using fluorescent microplate reader (Bio-Tek Instruments Winooski, VT, USA) at 530 nm and 590 nm. Cell viability was calculated using the ratio of the fluorescence of treated cells to the fluorescence of control cells, and the data were expressed as percentages.

Griess assay

The evaluation of NO production is performed through the measurement of the nitrite (NO_2^-) levels, the stable and nonvolatile breakdown products of NO with Griess reagent. After pretreatment with oxyresveratrol for 1 h, the cells irradiated with UVB were incubated for 24 h and then 100 µl of culture supernatant from each well was firstly added with 50 µl of the sulfanilamide solution and followed with 50 µl of the N-(1-napthyl) ethylenediamine hydrochloride (NED) solution. After incubation for 5-10 min in the dark at room temperature, the samples were measured using a microplate reader (Bio-Tek Instruments Winooski, VT, USA) at absorbance wavelength of 540 nm. The amount of nitrite level was calculated from the nitrite standard reference curve.

Intracellular ROS assay

Intracellular ROS production in HaCaT cells irradiated by UVB was studied through the oxidation of 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) to the fluorescent 2'-7'-dichlorofluorescein (DCF) (Invitrogen, CA, USA). Firstly, the cells were loaded with 20 µM of DCFH-DA and washed two times by 1X PBS. Then, the pretreated cells with oxyresveratrol for 1 h were irradiated by UVB irradiation (40 mJ/cm^2) and further incubated in a 37°C, 5% CO_2 incubator for 1.5 h. After that, the cells were immediately measured the fluorescent intensity by using a fluorescence microplate reader (Bio-Tek Instruments Winooski, VT, USA) with excitation wavelength of 485 and emission wavelength of 535 nm. The fluorescent signal intensity is a direct proportion to the ROS levels. H_2O_2 (100 µM) was used as a positive control for UVB-induced intracellular ROS formation.

Statistical analysis

Data are presented as mean±SEM of four independent experiments done in triplicates. The statistical significance was analyzed by one-way ANOVA (analysis of variance) and followed by Tukey's multiple comparison test using GraphPad Prism program version 5 (GraphPad software, Inc., San Diego, CA, USA). Differences were considered as statistically significant at $p<0.05$.

Results

The effect of oxyresveratrol on cell viability

Oxyresveratrol at the concentrations between 0.25-20 µM did not have cytotoxicity, while the high concentrations (>40 uM) became significantly toxic to the cells (**Fig 1A**).

The protective effect of oxyresveratrol on UVB-induced HaCaT cells damage

In **Fig 1B**, UVB irradiation decreased cell viability to 59.29±2.78% of control, while pretreatment with 2.5, 5, and 10 µM of oxyresveratrol significantly increase the cell survival to 67.49±1.61, 76.59±0.86, and 83.17±2.26% of control, respectively.

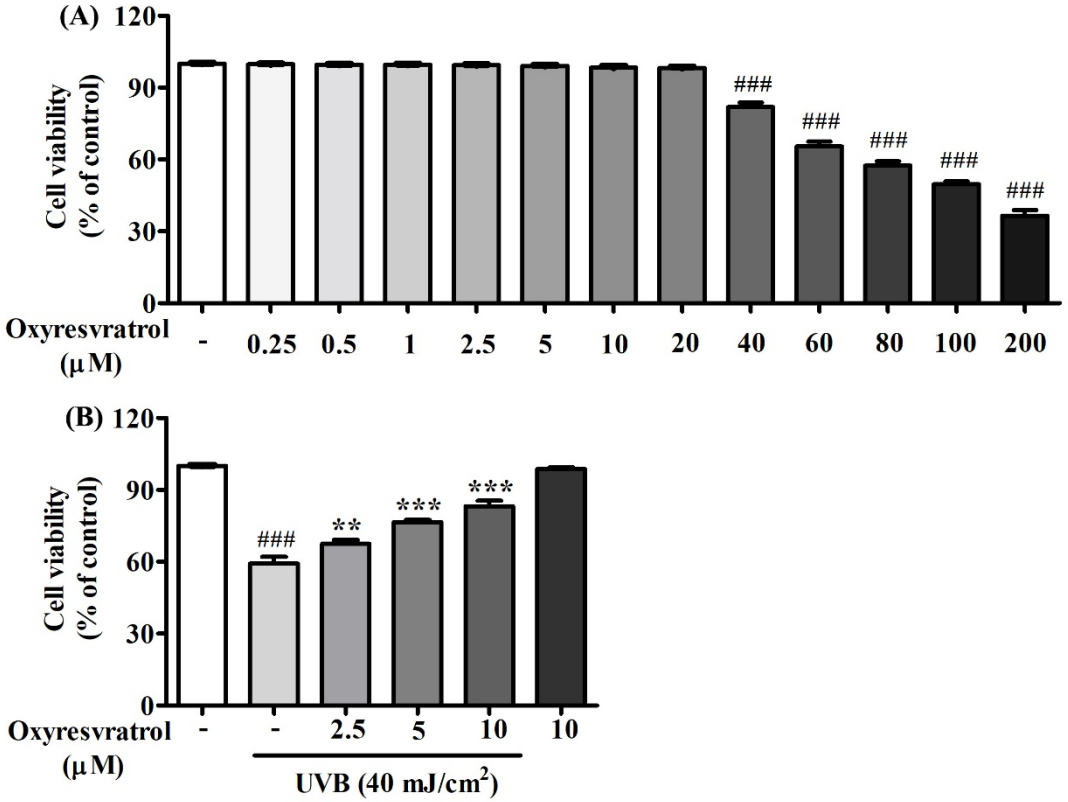

Figure 1. The effects of oxyresveratrol on HaCaT cell viability after UVB irradiation. The cells were treated with various concentrations of oxyresveratrol (0.25-200 uM) for 24 h incubation **(A)** or were pretreated with oxyresveratrol (2.5, 5, and 10 µM) for 1 h before exposure to UVB irradiation and further incubation for 24 h **(B)**. The cell viability was performed by using resazurin assay kit. Data were presented as a percentage to control. $^{###}p < 0.001$ as compared to the untreated cells; $^{**}p < 0.01$ and $^{***}p < 0.001$ as compared to UVB treatment alone.

The effect of oxyresveratrol on intracellular ROS production in UVB-irradiated HaCaT cells

In HaCaT cells, UVB radiation increased significant levels of intracellular ROS to 1.7±0.04 fold of control. The oxyresveratrol pretreatment at the concentrations of 2.5, 5, and 10 µM significantly decreased intracellular ROS levels in HaCaT cells after UVB irradiation to 1.18±0.05, 0.94±0.04, and 0.89±0.06 folds of control, respectively (**Fig 2.**).

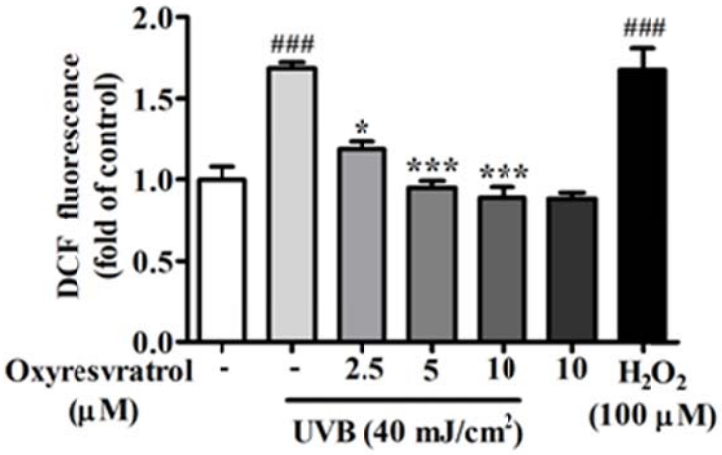

Figure 2. The effect of oxyresveratrol on UVB-induced intracellular ROS generation in HaCaT cells. The cells were pretreated with oxyresveratrol at the concentrations of 2.5, 5, and 10 uM for 1 h before exposure to UVB irradiation and further incubation for 1.5 h. H_2O_2 (100 μM) was used as a positive control. Data were expressed as a fold of control. $^{###}p < 0.001$ as compared to the untreated cells; *p< 0.05 and ***p< 0.001 as compared to UVB treatment alone.

The effect of oxyresveratrol on NO production in UVB-irradiated HaCaT cells

As shown in **Fig 3.**, after cells were irradiated with UVB, the radiation caused a significant increase in a level of nitrite to 19.82±1.17 μM whereas cells with oxyresveratrol at the concentrations of 2.5, 5, and 10 μM had significant decrease in the level of a concentration dependent manner to 16.00±0.52, 10.45±0.59, and 6.30±0.63 μM, respectively.

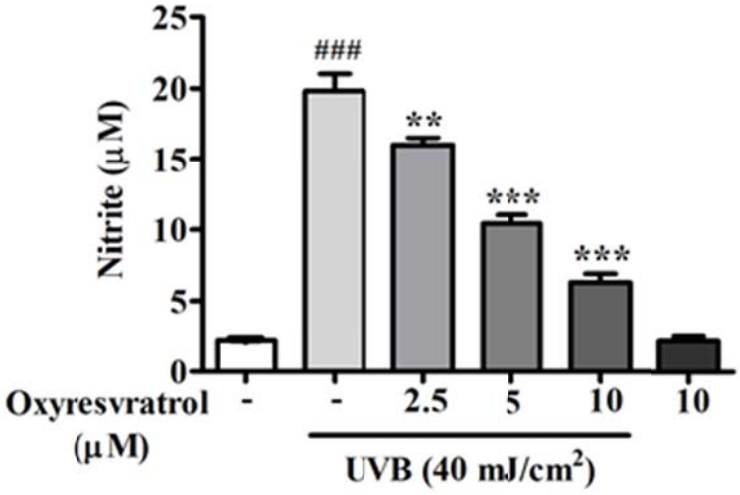

Figure 3. Effect of oxyresveratrol on UVB-induced NO production in HaCaT cells. The cells were pretreated with oxyresveratrol at the concentrations of 2.5, 5, and 10 uM for 1 h before exposure to UVB irradiation and further incubation for 24 h. The levels of the more stable NO metabolites, nitrite were investigated by using Griess assay. $^{###}p < 0.001$ as compared to the untreated cells (control); **p< 0.01 and ***p< 0.001 as compared to UVB treatment alone.

Discussion and Conclusion

ROS are chemically reactive chemical species such as hydrogen peroxide, superoxide, and hydroxyl radicals are produced as common intermediates of cellular metabolism and homeostasis (10). Overexpression of ROS results in the significant damage to cell structures. Excessive UVB exposure is one of the most crucial environmental risk factor for the development of skin disorders. The increased accumulation of ROS (11) and NO production (12) caused by UVB irradiation can trigger oxidative stress, eventually leading to skin inflammation (or sunburn) and skin cancer (13). In line with previous studies, UVB irradiation induced the increase of ROS and NO levels in HaCaT cells which consequently caused the cell death. A number of studies have supported that oxyresveratrol exerts high antioxidant activity and free radical scavengers (9,13,14). Consistently, pretreatment of HaCaT cells with oxyresveratrol significantly increased the cell viability in a concentration dependent manner. In parallel with the cell protective effect, oxyresveratrol strongly reduced both ROS and NO production in UVB-irradiated HaCaT cells, suggesting that the protective potential effect of oxyresveratrol might mediate through its free radical scavenging capacity. The present result was well correlated with that of Hu S and co-workers' research in a condition of UVA-exposed primary keratinocytes (15).

In conclusion, the present study revealed that oxyresveratrol isolated from the heartwood extract of *Artocarpus lakoocha* had the antioxidant activity by inhibiting the ROS and NO production in UVB-irradiated HaCaT cells. This effect contributed to protect keratinocyte HaCaT cells from the oxidative damage. Therefore, oxyresveratrol can be suggested as a potential natural antioxidant compound mixed in cosmetic products for preventing oxidative stress-induced skin deterioration from UVB radiation. However, further studies are needed to confirm benefit of the compound in other mechanisms.

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B08 Study of UGT3A1 T361G Nucleotide Polymorphism in Thai Muslims Living in Songkhla Province, Thailand

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Abstract

UDP-glucuronosyltransferase (UGTs) is the most important enzyme in phase II drug-metabolizing enzymes which consisted of 4 families such as UGT1 UGT2 UGT3 and UGT8. The UDP glycosyltransferases (UGT) attach sugar residues to small lipophilic chemicals to alter their biological properties and enhance elimination. UGT3 have 2 types are UGT3A1 and UGT3A2. UGT3A1 use UDP-*N*-acetylglucosamine to the sugar donor. This enzyme catalyzes the *N*-acetylglucosamine from UDP-*N*-acetylglucosamine to ursodeoxycholic acid, it is a member of the bile acids which has a role of lipid digestion. The researcher is aware of the importance of the mutation of the *UGT3A1* gene, which may one of the cause of abnormalities in the bile elimination process. It may cause gallstones in the gallbladder. Therefore, this study focuses on the study of *UGT3A1* gene polymorphism in muslim population representative in Songkhla province to see the frequency of genetic variation, use the information to describe the pathology of the bile elimination process. In this research, the researcher interested at the position 121 amino acid changing from Cysteine to Glycine causing an inactive enzyme [nucleotide 361T→G] (rs3756669), was determined on exon 4 (TGT →GGT) by using specific primer and PCR to increase the number of gene fragments required then detected the size of DNA by 1.5% agarose gel electrophoresis and nucleotide sequence would be determined by DNA sequencing. The 50 samples were analyzed, there were wild-type (T/T) 31 samples, 15 samples of heterozygous mutation (T/G) and 4 examples of homozygous mutation (G/G). So this study shows the populations in Songkhla Province have the potential for polymorphism at the amino acid position 121 is 38 percent. The genotype frequency were 0.620, 0.300 and 0.080, respectively and 0.770 (T) and 0.230 (G) allele frequency and the polymorphism at this position is under the Hardy-Weinberg equilibrium assumption. It can be concluded that, the genetic variation of the *UGT3A1* gene is very different from that of the Asian but close to the Caucasian. Therefore, the population in Songkhla Province may be having high risk for gallstones. However, the *UGT3A1* gene should be screened before treatment with drug that causes gallstone adverse drug reaction and use for the artherosclerosis marker.

Keywords: UDP-glucuronosyltransferase (UGTs), polymorphism, allele frequency, Hardy-Weinberg law

การศึกษาการกลายพันธุ์ของยีน *UGT3A1* ที่ตำแหน่ง T361G ในตัวแทนประชากรมุสลิมในจังหวัดสงขลา

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บทคัดย่อ

UDP-glucuronosyltransferase (UGTs) เป็นเอนไซม์ใน Phase II metabolism ทำให้สารมีคุณสมบัติละลายน้ำได้ดี พร้อมที่จะถูกขับออกจากร่างกาย ในมนุษย์พบ 4 families ได้แก่ UGT1, UGT2, UGT3 และ UGT8 สำหรับ UDP-glycosyltransferase 3 (UGT3) มี 2 ชนิด คือ UGT3A1 และ UGT3A2 substrates หลักของ UGT3A1 คือ ursodeoxycholic acid (UDCA) ซึ่งเป็นสารในกลุ่ม bile acids ซึ่งมีบทบาทสำคัญในการย่อยอาหารประเภทไขมัน ผู้วิจัยจึงมีความสนใจในการศึกษาความถี่ของการกลายพันธุ์ของยีน *UGT3A1* ในประชากรชาวมุสลิมในจังหวัดสงขลา เนื่องจากการกลายพันธุ์ของยีนอาจจะเป็นสาเหตุหนึ่งในการทำให้เกิดโรคนิ่วในถุงน้ำดี ดังนั้นจุดประสงค์ของงานวิจัยนี้ เพื่อศึกษาความถี่ในการเกิดความผันแปรทางพันธุกรรมและนำข้อมูลไปใช้ในการอธิบายการเกิดพยาธิสภาพเกี่ยวกับกระบวนการสร้างและทำลายน้ำดี ในงานวิจัยนี้ทางผู้วิจัยได้สนใจตำแหน่งที่จะศึกษาการกลายพันธุ์ที่ตำแหน่งกรดอะมิโนที่ 121 อยู่บน exon 4 เปลี่ยนจาก Cys121Gly นิวคลีโอไทด์ที่ 361T→G (rs3756669) ส่งผลให้เอนไซม์ไม่สามารถทำงานได้ วิธีการศึกษาโดยการสุ่มตัวอย่าง genomic DNA จากชาวมุสลิมในจังหวัดสงขลาจำนวน 50 ตัวอย่าง นำมาทำ PCR ด้วย primer ที่ออกแบบเพื่อเพิ่มจำนวนชิ้นส่วนของยีนที่ต้องการแล้วนำไปตรวจสอบขนาดของชิ้นส่วนยีน ด้วยวิธี 1.5% agarose gel electrophoresis และส่งตรวจวิเคราะห์ลำดับเบส (DNA sequencing) พบว่าตัวอย่างที่สุ่มมาศึกษาการกลายพันธุ์ที่ตำแหน่งดังกล่าว มีสมดุลตามกฎของ Hardy-Weinberg และ มีตัวอย่างที่เป็นยีนแบบ wild-type (T/T), heterozygous mutation (T/G) และ homozygous mutation (G/G) จำนวน 31, 15 และ 4 ตัวอย่างตามลำดับ คิดเป็นความถี่จีโนไทป์เท่ากับ 0.620, 0.300 และ 0.080 ตามลำดับ และมีความถี่แอลลีลเท่ากับ 0.770 (T) และ 0.230 (G) สามารถสรุปได้ว่าความถี่จีโนไทป์ ความถี่แอลลีลของความแปรผันทางพันธุกรรมของยีน *UGT3A1* มีความแตกต่างกับประชากรในแถบเอเชียแต่มีความใกล้เคียงกับประชากรคอเคเชียน ดังนั้นในประชากรในจังหวัดสงขลาอาจมีความเสี่ยงในการเกิดโรคนิ่วในถุงน้ำดี อย่างไรก็ตามควรมีการตรวจยีน *UGT3A1* ก่อนการรักษาด้วยยาที่มีอาการไม่พึงประสงค์ที่ทำให้เกิดโรคนิ่วถุงน้ำดีหรืออาจใช้ยีนนี้เป็น marker ของ artherosclerosis ในอนาคต

คำสำคัญ : UDP- glycosyltransferase (UGTs), การกลายพันธุ์, allele frequency, Hardy-Weinberg

Introduction

Drug metabolism is a process of changing the chemical molecule of drug to provide good solubility. Those molecule are polar and excret in urine and bile easily. The drug metabolism is divided into 2 phases, phase I metabolism and phase II metabolism **(**1**).** Phase I is the major process of oxidation, reduction and hydrolysis by cytochrome P45. Phase II is the conjugation process. The most common enzyme in phase II is UDP-glucuronosyltransferases (UGTs) **(**2**).** Currently, UGTs are classified into 4 main families, UGT1, UGT2, UGT3 and UGT8 (3). UGT3 have two types, UGT3A1 and UGT3A2. The main substrates of UGT3A1 are ursodeoxycholic acid (UDCA), a substance in the bile acids (4). The amount of bile acids absorption will affect to the rate of bile acid synthesis in the liver. Some bile acids are not reabsorbed but there are excreted in the feces. Bile acids play a role in eliminating cholesterol from the body through the feces (5). So UGT3A1 maybe useful as a predictor of atherosclerosis risk (4). The study in the past, the genetic variation of the *UGT3A1* gene in the position of the amino acid at position 121 by switching from TGT (cysteine) to GGT (glycine) cause the inactive protein (4). According to a recent report, the genetic variation at this location is about 11% for the Asian population and about 20% for the Caucasians (4). Based on the data, the researcher is aware the importance of the polymorphism of the *UGT3A1* gene, which cause adverse reactions to medications that are metabolised by abnormal enzymes or cause the abnormality in the bile process and increase the atherosclerosis risk because 7β-Hydroxycholesterol (steroids) is good substrates of UGT3A1. It is the main toxic products of cholesterol and induces macrophages at the plaque of the arteries (4). The genotype of *UGT3A1* polymorphism in Thailand and Southeast Asian countries has not been conducted yet. This study focuses on the genetic variation of *UGT3A1* gene in Muslim population in Songkhla province to see the frequency of genetic variation, use the information to describe the pathology of the bile process and in the future, it is expect to be used to prevent adverse drug reactions from drug that are metabolized by abnormal UGT3A1 enzyme.

Materials and Methods

50 Newborn cord blood samples were collected and approved under human ethic permission code SUB.EC 52-231-19-2-3 from Human Research Ethics Committee (HREC), Faculty of Medicine, Prince of Songkla University. 50 Genomic DNAs were extracted from newborn cord blood samples which were collected randomly at Songkhla hospital in Songkhla province via QIAGEN DNA extraction kit.

Known polymorphism of *UGT3A1* gene is 361T→G (rs3756669) caused Cys121Gly was focused in this study and primer design was performed by using the sequence of *UGT3A1* gene including promoter, intron and exon. The primers were analyzed by the NCBI program database. (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi). The forward primer is 5'GGCTCCCAGAGTAGGACGTT 3' and the reverse primer is 5'TTCAACCTCAGGATCCCACTG 3'. The 20 ul PCR reaction consists of 2 µl *Tag* buffer, 1.4 µl $MgCl_2$, 2 µl primer mix, 0.5 µl dNTPs, *Taq* polymerase 0.5 µl, ddH_2O 12.6 µl, and DNA template 1 µl. For PCR condition, 94.0 ºC 4 minutes 1 cycle, 30 cycles of 94.0 ºC 30 seconds, 58.1 ºC 30 seconds and 72.0 ºC 1 minute, follows by 72.0 ºC 5 minutes and 6.0 ºC 5 minutes. Then the sample was confirmed the correct size on 1.5% agarose gel electrophoresis sequencing by First Base laboratories SDN BHD Malaysia

Data analysis The Hardy-Weinberg law and allele frequency were calculated by SNPAlyze version 9.0 (trial version) (https://www.dynacom.co.jp/english/snpalyze_e/e_sa_demo.html)

Results

Genotype of UGT3A1 gene of newborn genomic DNA sample from Songkhla hospital

A total of 50 genomic DNA samples were obtained randomly from newborn cord bloods which were collected at Songkhla hospital and extracted by QIAGEN genomic DNA extraction kit. The PCR was performed to get the product size of *UGT3A1* gene fragment at 904 bps on 1.5% agarose gel electrophoresis and then the nucleotide sequencing were done and the polymorphism was checked comparing with wild-type sequence (rs3756669)

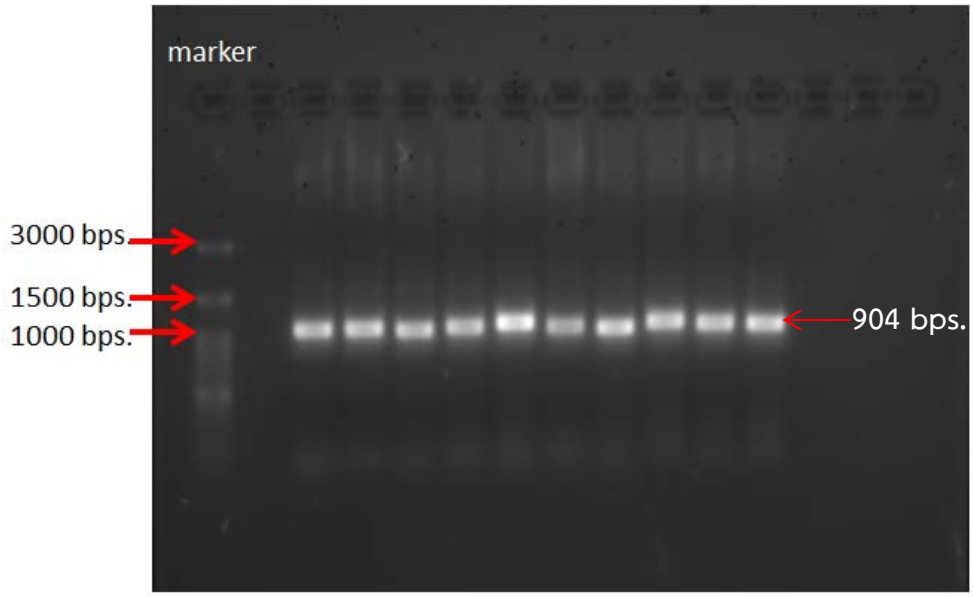


Figure 2. The picture shows the fragment size of PCR product of *UGT3A1* was 904 bps.

Mutation of UGT3A1 gene at Cys121Gly

The result shows the number of wild-type, heterozygous and homozygous are 31, 15 and 4. The genotype frequency is wild type (T/T) (0.620), heterozygous mutation (T/G) (0.3) and homozygous mutation (G/G) (0.08). The allele frequency of *UGT3A1* variants are 361T = 0.770 and 361G = 0.230. (Table 1)

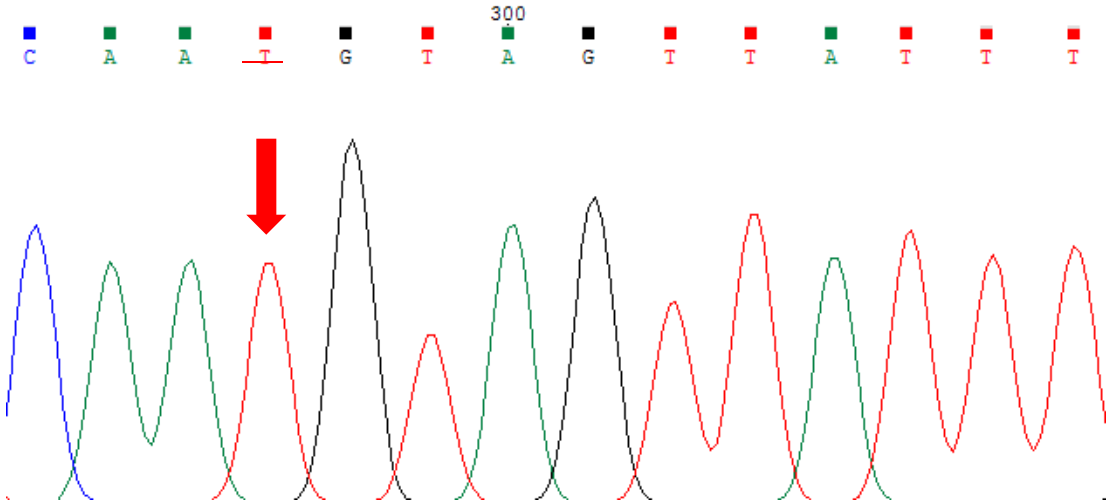
The *UGT3A1* gene polymorphism at 361T>G on exon 4 are under the Hardy-Weinberg law (p>0.05)

The wild-type and polymorphism points both heterozygous and homozygous are displayed in the electropherograms in Figure 1-2.

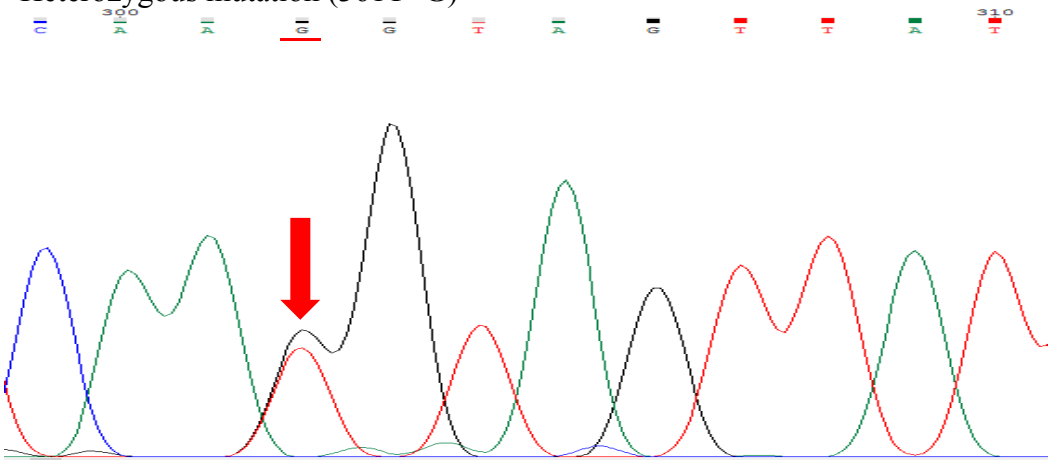
Table 1. Genotype frequency of *UGT3A1* gene polymorphism. (n=50)

Polymorphism of *UGT3A 1* gene	Genotype frequency	Allele frequency	Observed	Expected	Chi-square
361T>G T/T	0.620	T = 0.77 G = 0.23	31	29.65	$X^2 = 1.17$
T/G	0.300		15	17.71	
G/G	0.080		4	2.65	

A. Wild type



B. Heterozygous mutation (361T>G)



C. Homozygous mutation (361T>G)

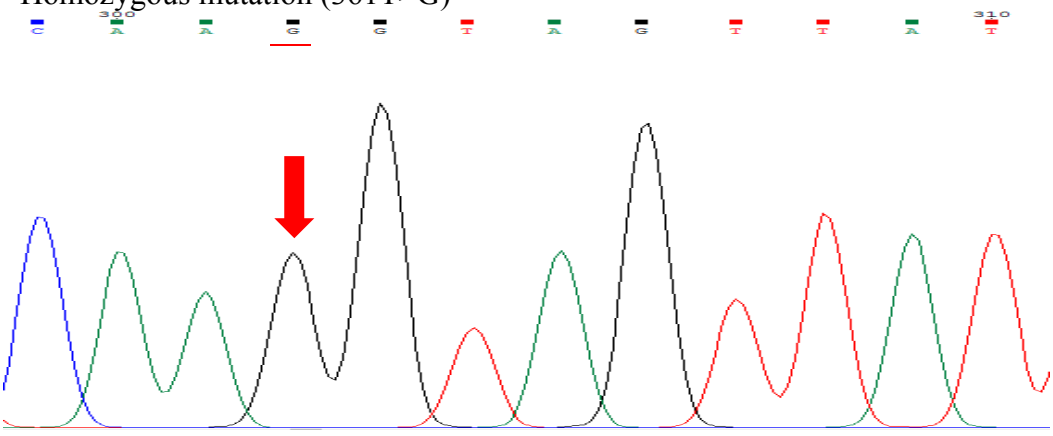


Figure 2. Electropherograms in exon4 at the position 361 nucleotide sequence of the *UGT3A1* gene. A: Wild type, B: heterozygous mutation and C: homozygous mutation

Discussion

In this research, the muslim population in Songkhla province are representative of southern of Thailand. There are no relocation and conversion three generations before the population representative. Samples were studied newborn baby from Thai pregnant women who are Thai muslim nationality and birth at Songkhla Hospital, Songkhla since November 2009 to March 2010.

From the study of gene polymorphism at 121 amino acid sequence (361 nucleotide) on exon4 of the *UGT3A1* gene in 50 southern Thai Muslim neonates are found 15 heterozygous mutations and 4 homozygous mutations. No found a new polymorphism at this gene fragment. The polymorphism at Cys121Gly was found to be known polymorphism when comparing the allele frequencies of the *UGT3A1* gene polymorphism to the previously reported ethnic populations. Studying in Asian populations (Japanese) were estimated at about 0.110 and the Caucasian population is about 0.200 (4) but not found in African and American populations **(**6**).** The alleles frequency of *UGT3A1* gene variant in this study was higher than that of Asian population. It is close to the Caucasian population. Therefore, genetic diversity of genes in different populations should be studied. Although there is already information in that region but it is not represent to all people in every country in muslim region. In the Hardy-Weinberg analysis, the gene polymorphism at this location is under an assumption of Hardy-Weinberg rule. These samples used in this study are Thai Muslim. The most they were married among Thai Muslims together. Thus, the genetic is not change much from generation to generation.

The polymorphism at this location is missense polymorphism, a polymorphism of the nucleotide gene that changes the mRNA codon and changes the amino acid translation. **(**7**)** It making the enzyme inactive **(**4**)**. Nowadays, ursodeoxycholic acid is the only bile acids drug use to treat liver dysfunction in patients with gallstones (8, 9). For example, when ursodeoxycholic acid is given to patients with bile disease in the range of 10-15 mg / kg for 2-3 weeks, it is found that 50% of all patients have decreased bile acid content within the blood 3-4% (10). Under this condition, ursodeoxycholic acid is increased and excreted by the UGT3A1 enzyme that using co-substrate as *N*-acetylglucosamine. 7β-hydroxyl group of the substrate combine with co-substrate and then excreted outside the body (11,12). In addition, 7β-Hydroxycholesterol (steroids) is another substrate that reacts with *UGT3A1*. It is the one of main toxic products of cholesterol. 7β-hydroxycholesterol which may induce macrophages at the plaque of the arteries and causes artherosclerosis (13). From the above information, it can be seen the polymorphism at position 361T>G will result in the population being treated. Ursodeoxycholic acid may not work and cause adverse reactions. It may also cause the artherosclerosis, this can be fatal. Future study should include more sample size, more other region in Thailand relationship between *UGT3A1* polymorphism with artherosclerosis incidence and ursodeoxycholic acid adverse drug reaction. Therefore, the detection of *UGT3A1* gene polymorphism at various sites prior to substrate therapy has the potential to achieve the highest therapeutic efficacy and to reduce the risk of adverse drug reactions.

Conclusion

A total of 50 samples of muslim Thais in Songkhla province show the polymorphism of *UGT3A1* gene at 361T>G have 38% of all samples. The genotype frequency of the wild-type (T/T) heterozygous mutation (T/G) and homozygous mutation (G/G) were 0.620, 0.300 and 0.080, respectively. Alleles frequency of the *UGT3A1* polymorphism were 0.770 (T) and 0.230 (G). The polymorphism of the *UGT3A1* gene at 361T>G cause abnormal protein formation or loss of enzyme activity. The inactive enzyme may result in abnormalities in the bile drainage process or the adverse effects of UGT3A1 substrates and it may also cause high artherosclerosis risk than normal. However, the *UGT3A1* gene should be screened before treatment with drug to reduce the risk of adverse reactions and use for the artherosclerosis marker.

Acknowledgements

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B09 Increased Nitrite in Breast Cancer Tissues from Patients

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Abstract

Breast cancer is a common cancer in women. Breast cancer is categorized with basis on expression of hormonal receptors, including estrogen receptor (ER) and progesterone receptor (PR). Nitric oxide (NO) has been reported to promote cancer development. NO is ubiquitously stored in tissues as nitrite at micromolar range, which can be reduced to NO under hypoxia and acidosis. This study aimed to investigate nitrite, nitrate levels and mRNA expression of nitric oxide synthases (NOS) in ER-positive, ER-negative breast cancer tissues compared to normal adjacent tissues from forty-five breast cancer patients**.** The NO levels in breast cancer by measuring nitrite and nitrate levels by tri-iodide and vanadium chloride based chemiluminescense method. The mRNA expression of NOS enzymes was determined by real-time polymerase chain reaction (real-time PCR). We found that nitrite levels were higher in ER-positive and ER-negative breast cancer than normal adjacent breast tissues. ER-negative breast cancer exhibited higher nitrite levels than ER-positive breast cancer. The nitrate levels were not different between cancer and normal tissues. mRNA expressions of endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS) were not different between cancer and adjacent tissues. We conclude that breast cancer has increased nitrite levels, in particular in ER-negative breast cancer. More patients with ER- negative breast cancer are needed to test the association of nitrite with disease progression.

Keywords: nitrite, breast cancer, estrogen receptor

การเพิ่มขึ้นของไนไตรท์ในเนื้อเยื่อมะเร็งเต้านมจากผู้ป่วย

พีรวิชญ์ คงแก้ว[1], นันท์นภัส เพียวพงศ์[2], รณรัฐ สิวิกะปกรณ์กุล[3], พรพรรณ วิวิธนาภรณ์[1], ณัฐวุธ สิบหมู่[1]

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บทคัดย่อ

มะเร็งเต้านมเป็นมะเร็งที่พบได้บ่อยในผู้หญิง มะเร็งเต้านมสามารถแบ่งกลุ่มได้ตามตัวรับของฮอร์โมนได้แก่ ตัวรับเอสโตรเจน (ER) และตัวรับโปรเจสเตอโรน (PR) ไนตริกออกไซด์ (NO) ได้มีการรายงานว่าสามารถส่งเสริมการพัฒนาของมะเร็ง ไนตริกออกไซด์โดยปกติจะถูกเก็บในเนื้อเยื่อรูปของไนไตรท์ในระดับไมโครโมลาร์ ไนไตรท์สามารถเปลี่ยนเป็นไนตริกออกไซด์ได้ภายใต้ออกซิเจนต่ำและภาวะเลือดเป็นกรด เป้าหมายของการศึกษาคือ การวัดระดับไนไตรท์และไนเตรต และการแสดงออกของเอ็มอาร์เอนเอของไนตริกออกไซด์ซินเทสในเนื้อเยื่อมะเร็งเต้านมจากผู้ป่วยทั้งชนิดมีตัวรับเอสโตรเจนและไม่มีตัวรับเอสโตรเจนเปรียบเทียบกับเนื้อเยื่อปกติข้างเคียง จากผู้ป่วย 45 คน ระดับไนตริกออกไซด์ในมะเร็งเต้านมโดยการวัดระดับไนไตรท์และไนเตรต โดยใช้ไตรไอโอไดด์และวานาเดียม โดยวิธีเคมิลูมิเนสเซนส์ การแสดงออกของเอ็มอาร์เอนเอของไนตริกออกไซด์ซินเทส โดยเรียลไทม์ พีซีอาร์ ผลการทดลองพบว่าระดับของไนไตรท์สูงในเนื้อเยื่อมะเร็งเต้านมทั้งชนิดมีตัวรับเอสโตรเจนและไม่มีตัวรับเอสโตรเจนกว่าเนื้อเยื่อปกติใกล้เคียงมะเร็งระดับไนเตรตไม่มีการแตกต่างกันในเนื้อเยื่อมะเร็งและเนื้อเนื่อปกติ การแสดงออกของเอ็มอาร์เอนเอของเอนโดทีเลียวไนตริกออกไซด์ซินเทส (eNOS) และอินดิวซิเบิลไนตริกออกไซด์ซินเทส (iNOS) ไม่มีความแตกต่างกันในเนื้อเยื่อมะเร็งและเนื้อเยื่อปกติข้างเคียง สรุปได้ว่ามะเร็งเต้านมมีระดับไนไตรท์ที่สูงขึ้นโดยเฉพาะอย่างยิ่งในมะเร็งเต้านมชนิดไม่มีตัวรับเอสโตรเจน จำเป็นที่จะต้องเพิ่มจำนวนผู้ป่วยมะเร็งเต้านมชนิดไม่มีตัวรับเอสโตรเจนเพื่อหาความสัมพันธ์ระหว่างไนไตรท์และการดำเนินไปของโรค

คำสำคัญ: ไนไตรท์, มะเร็งเต้านม,ตัวรับเอสโตรเจน

Introduction

Breast cancer is a common cancer in women. Breast cancer is categorized with basis on hormonal receptors on tumor cells including estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). Expression of hormone receptors is used to guide treatment. ER-positive breast cancer which is more common than ER-negative breast cancer is treated with anti-hormone therapy. In contrast, ER-negative breast cancer is more difficult to be treated because of lack of target, and has poor prognosis.

Nitric oxide (NO) has multiple roles in physiological and pathological processes such as vasodilation, platelet inhibition, promotion of cell proliferation and growth, angiogenesis, or tumorigenesis (1). NO is produced from nitric oxide synthase (NOS), and stored in many tissues as nitrite and S-nitrothiols. Expressions of NOS mRNA including endothelium nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS) are increased in breast cancer. NO has dual effects on breast cancer (2). NO at low concentration can inhibit apoptosis and promote angiogenesis and proliferation of cancer cells. In contrast, NO at high concentration can induce apoptosis of cancer cells. NO may be a target for cancer therapy; for example inhibition of eNOS-derived NO may inhibit cancer cell growth.

This study aimed to investigate nitrite, nitrate levels and mRNA expression of nitric oxide synthases in ER-positive, ER-negative breast cancer tissues compared to normal adjacent tissues from forty-five breast cancer patients.

Materials and Methods

Tissues collection

Normal adjacent and cancer of breast tissues from patients were obtained from Department of Surgery, Ramathibodi Hospital, Mahidol University, Thailand. Breast cancer tissues were collected from operation room and stored at -80 ^{o}C until use. The ER status was reported by Department of Pathology, Ramathibodi Hospital, Mahidol University, Thailand. Clinical stage was categorized by TMN staging from the medical record. The project was approved by Committee on Human Rights Related to Research Involving Human Subjects based on the declaration of Helsinki, protocol ID 03-58-32.

Measurement of nitrite and nitrate

Frozen tissues were thawed on ice and weighted before homogenization. Tissues were homogenized with 1.5 ml of homogenized solution containing nitrite stabilizers (1 mM of KCN, 12mM of $K_3Fe_2(CN)_6$, 10 mM of NEM and 100 μM of DTPA) (3). Afterwards, homogenized tissues were injected into purge vessel containing tri-iodide solution or vanadium chloride. The signals were recorded and area under the curve (AUC) was determined using Origin 7 (Origin lab, USA).

Real-time polymerase chain reaction (real-time PCR)

Breast cancer tissues were homogenized with lysis buffer containing 2-mercaptoethamol (Jena Bioscience, Germany). The RNA extraction and real-time PCR were prepared as previously described (4). The primer sequence of human eNOS were: forward 5'-ACCCTCACCGCTACAACATC -3' and reverse 5'- GCCTTCTGCTCATTCTCCAG -3'and human iNOS were: forward 5'- ACAACAAATTCAGGTACGCTGTG -3' and reverse 5'-TCTGATCAATGTCATGAGCAAAGG -3'.

Statistical analysis

Data were presented as median with interquartile range (IQR). Comparison was done by Mann Whitney *U* test, Wilcoxon signed rank test, or Kruskal-Wallis test. *p* value less than 0.05 was considered statistically significant difference. Statistical analysis was performed using GraphPad Prism version 6.

Results

Patients' clinical data

Table 1. General characteristics of breast cancer patients

Characteristics	Number of patients
Total	45
Age (years, median(IQR))	53(45 - 63)
ER status	
• Positive	32
• Negative	13
Clinical stage	
• Stage 1 (T1N0M0, T2N0M0, T1N1M0)	23
• Stage 2 (T2N1M0, T1N2M0, T2N2M0)	15
• Stage 3 (T2N3M0, T3N1M0, T4N0M0, T4N1M0)	7

Note: ER = estrogen receptor, M = distant metastasis, N = lymph node, T = tumor size

Levels of nitrite in breast cancer tissues

Nitrite levels were reported in nanomole per gram of breast tissue. The nitrite levels in cancer tissues were higher than normal adjacent breast tissues [median (IQR): 8.76(1.66 – 20.82) vs 6.91(2.43 – 12.31), n=45] (Figure 1A). The nitrite levels in ER-positive breast cancer tissues were higher than those in normal adjacent tissues [median (IQR): 6.82(1.49 – 16.81) vs 5.76(1.13 – 9.14), n=32] (Figure 1B). The nitrite levels in ER-negative breast cancer tissues were also higher than those in normal adjacent tissues [median (IQR): 24.30(4.83 – 33.26) vs 13.71(7.63 – 20.16), n=13] (Figure 1C). The nitrite levels in ER-negative breast cancer tissues were higher than ER-positive breast cancer tissues [median (IQR): 24.30(4.83 – 33.36) vs 6.82(1.49 – 16.81] (Figure 1D).

Levels of nitrate in breast cancer tissues

Nitrate levels were reported in nanomole per gram of breast tissues. The nitrate levels in cancer tissues and normal adjacent tissues was not different (Figure 2A). There was no difference in nitrate levels between ER-positive vs normal adjacent tissues (Figure 2B), ER-negative vs normal adjacent tissues (Figure 2C), and ER-positive vs ER-negative breast cancer tissues (Figure 2D).

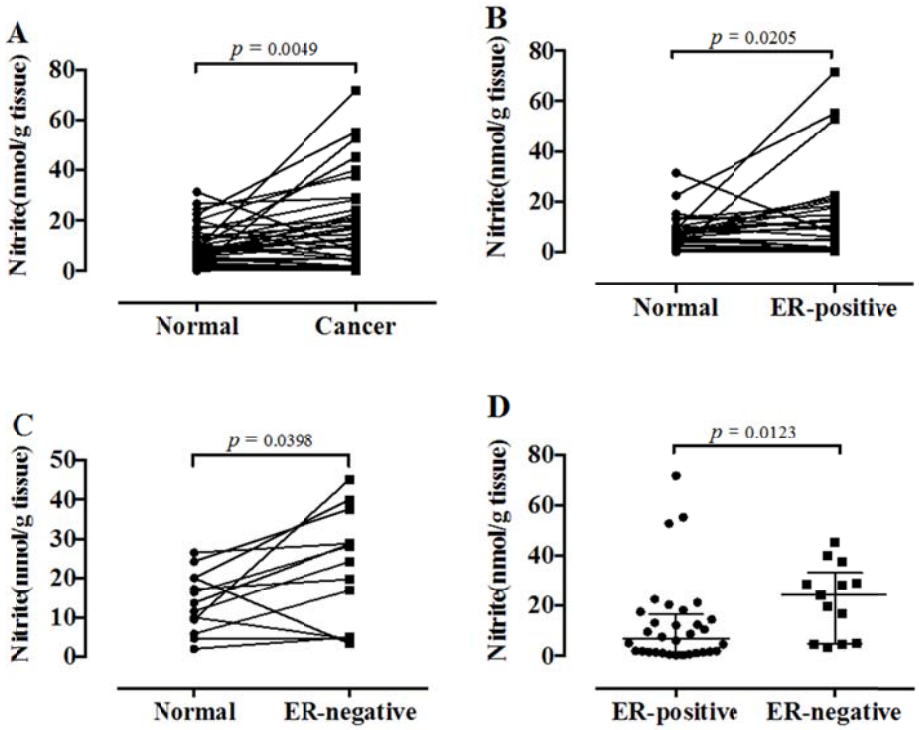

Figure 1. Nitrite levels in breast cancer and normal adjacent tissues. (A) Cancer tissues had higher nitrite levels than normal adjacent tissues (n=45). ER-positive breast cancer tissues (n=32) (B) and ER-negative breast tissues (n=13) (C) had higher nitrite levels than normal adjacent tissues. ER-negative breast cancer (n=13) had higher nitrite levels than ER-positive (n=32) breast cancer (D). Comparison was done by Wilcoxon signed rank test and Mann Whitney *U* test.

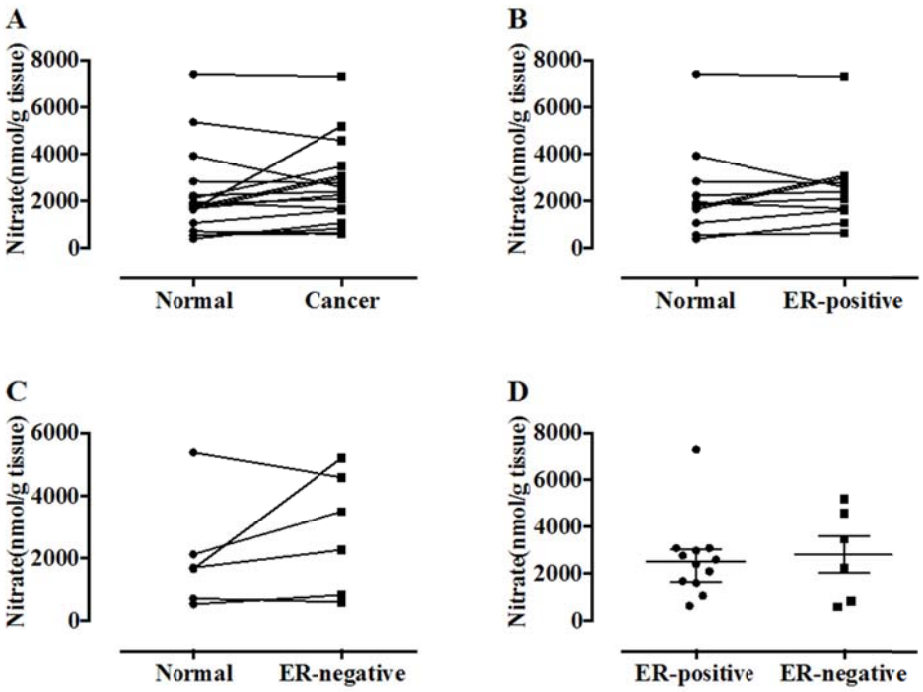

Figure 2 Nitrate levels in breast cancer and normal adjacent tissues. Nitrate levels were compared between breast cancer tissues vs normal adjacent tissues (n=18) (A), ER-positive breast cancer tissues vs normal adjacent tissues (n=12) (B), ER-negative breast cancer tissues vs normal adjacent tissues (n=6) (C), and ER-positive (n=12) vs ER-negative (n=6) breast cancer tissues (D). Comparison was done by Mann Whitney *U* test.

Levels of nitrite and nitrate in breast cancer tissues at different stages

The levels of nitrite and nitrate were reported in nanomole per gram of breast tissue categorized in clinical stage 1, 2 and 3 of breast cancer showed no difference in any stage of breast cancer.

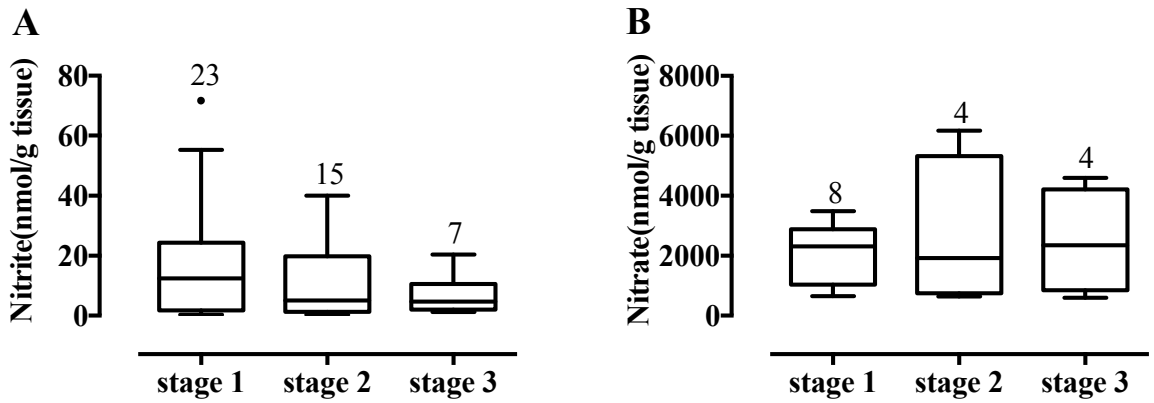

Figure 3 Box plot of nitrite (A) and nitrate (B) levels in breast cancer tissues at different stages. Whiskers indicate 1.5xIQR and minimal values. There was no difference in nitrite and nitrate among stages (Kruskal-Wallis test). Number of cases are indicated above the boxes.

mRNA expression of NOS in breast cancer tissues

The mRNA expressions of eNOS and iNOS were analyzed by real-time PCR. There was no difference in eNOS and iNOS mRNA expression between normal adjacent and cancer tissues (Figure 4).

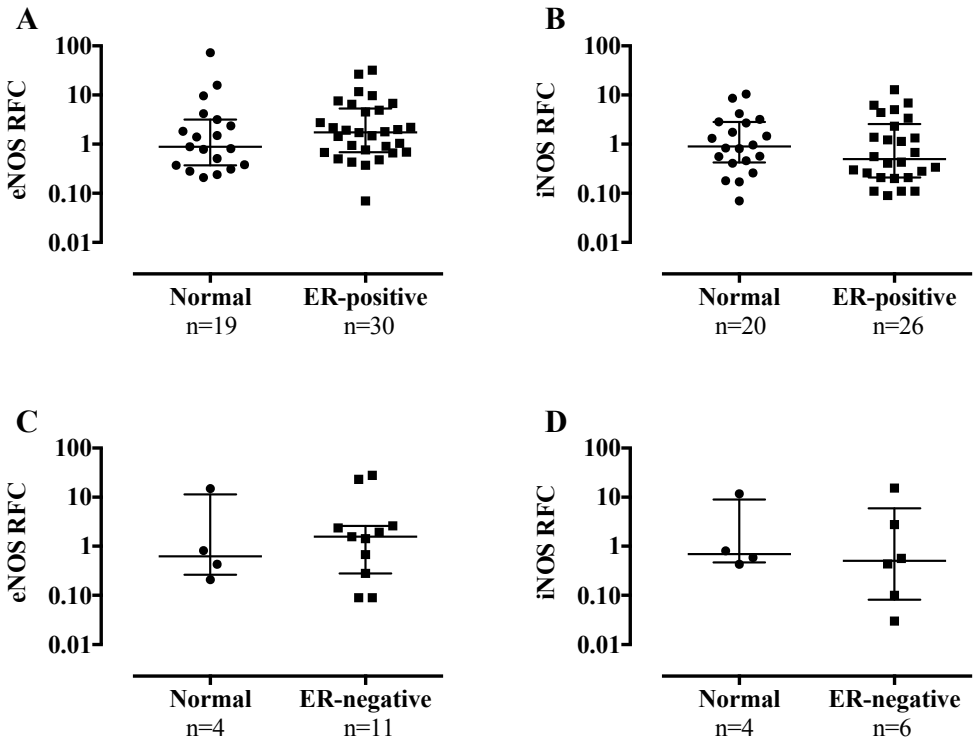

Figure 4 Relative fold change (RFC) of mRNA expression of eNOS and iNOS in ER-positive (A and B) and ER-negative (C and D) breast cancer tissues from patients. Lines are medians and IQR. Comparison was done by Mann Whitney *U* test.

Discussion

In this study, we found the nitrite levels in breast cancer tissues were higher than normal adjacent tissues in both ER-positive and ER-negative breast cancer tissues. The nitrite levels in ER-negative breast cancer are higher than ER-positive breast cancer. Nitrite is a storage form of nitric oxide in tissues; for example the nitrite levels in liver and skeletal muscle are 2.5 and 4.5 nmol/g tissue, respectively (5) which are in the same concentration range as our data. Nitrite is reduced to nitric oxide by deoxygenated heme proteins under hypoxia and acidosis, a condition found in tumor microenvironment. Nitric oxide is essential for cancer. Nitric oxide at concentrations in range of 10 – 20 nM promotes cancer cell proliferation, growth, angiogenesis, and metastasis (6). Therefore, high nitrite levels serve as reservoir of nitric oxide in cancer cells. No change in nitrate levels is consistent with the fact that nitrate is an inert product, which cannot be reduced to nitrite or nitric oxide.

ER-negative breast cancer has higher nitrite than ER-positive cancer, which corresponds with more aggressive behavior of ER-negative cancer. Despite increased nitrite in breast cancer tissue, the nitrite levels in different stages of cancer are not different, suggesting that nitrite is not indicator for severity. Nitrate levels were not different between normal adjacent and cancer tissues, and between ER-positive and ER-negative breast cancer tissues suggesting nitrate was not associated with cancer. Previous study with immunohistochemistry showed no correlation of eNOS with tumor size and grade (7, 8). Regulation of eNOS activity is modulated by post-translational mechanism such as phosphorylation and calcium, while iNOS regulation is at the transcriptional level. No change in eNOS and iNOS mRNA represents no alteration in transcription of these enzymes. To elucidate the mechanism of increased nitrite, regulation of eNOS at post-translational levels should be investigated in the future. The correlation of nitrite and clinical progression is ongoing. Further investigation should be performed to assess nitrite as biomarker for prognosis.

Conclusion

Nitrite level is higher in both ER-positive and ER-negative cancer than normal adjacent breast tissues. In addition, ER-negative breast cancer has higher nitrite than ER-positive cancer. There is no difference in nitrate. Cancer at different stages has no difference in nitrite. The mRNA expression of eNOS and iNOS are not different in ER-positive and ER-negative. NO could be a biomarker and therapeutic target for breast cancer in the future.

Acknowledgements

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B10 The Association of *ABCB1* C1236T with Molecular Response to Imatinib in Thai Patients with Chronic Myeloid Leukemia

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Abstract

Imatinib has been shown to be a substrate of efflux transporter including P-glycoprotein (P-gp). Consequently, the polymorphisms in the gene encoding P-gp may influence on clinical outcomes of imatinib therapy. However, these topics are still inconclusive and no studies have been done in Thai patients with CML. Therefore, the aim of this study was to determine the association of *ABCB1* C1236T with clinical outcomes of imatinib in Thai patients with CML. This study used an observational retrospective design. Ninety-five Thai patients with CML treated with imatinib at Ramathibodi Hospital between 2001 and 2017 were studied. Clinical data and blood samples were collected. Genomics DNA were genotyped. For statistical analysis, the Hardy-Weinberg equilibrium and the association of *ABCB1* C1236T with clinical outcomes were determined by Chi-square test. The results showed that all genotype frequencies including homozygous CC, heterozygous CT and homozygous TT were in accordance with Hardy-Weinberg equilibrium (CC = 12.63%, CT = 52.63 % and TT = 34.74%). *ABCB1* C1236T was associated with molecular response in Thai patients with CML. Patients with genotypes of CC or CT had a significantly lower rate of major molecular response (BCR-ABL1^{IS}≤ 0.1%)/complete molecular response (undetected level of BCR-ABL transcripts) compared to those with TT genotype (OR = 7.68, 95%CI = 0.952-61.949, p = 0.030). The result suggests the influence of *ABCB1* C1236T on clinical outcomes of imatinib in Thai patients with CML.

Keywords: *ABCB1* C1236T, imatinib, chronic myeloid leukemia, molecular response

ความสัมพันธ์ของ *ABCB1* C1236T กับการตอบสนองระดับอณูของยาอิมมาทินิบในผู้ป่วยโรคมะเร็งเม็ดเลือดขาวเรื้อรังชนิดมัยอีลอยด์ชาวไทย

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บทคัดย่อ

ยาอิมมาทินิบมีคุณสมบัติเป็นซับสเตรตของโปรตีนผลักยาออกจากเซลล์ชนิด P-glycoprotein (P-gp) การเกิดภาวะพหุสัณฐานของยีนที่สร้างโปรตีน P-gp (*ABCB1*) อาจส่งผลต่อการรักษาทางคลินิกของยาอิมมาทินิบ อย่างไรก็ตามยังไม่มีข้อสรุปชัดเจนในประเด็นดังกล่าว และยังไม่มีการศึกษาในผู้ป่วยโรคมะเร็งเม็ดเลือดขาวเรื้อรังชนิดมัยอีลอยด์ชาวไทย ดังนั้นการศึกษานี้จึงมีวัตถุประสงค์เพื่อหาความสัมพันธ์ของ *ABCB1* C1236T กับการตอบสนองระดับอณูของยาอิมมาทินิบในผู้ป่วยโรคมะเร็งเม็ดเลือดขาวเรื้อรังชนิดมัยอีลอยด์ชาวไทย โดยมีรูปแบบการศึกษาแบบสังเกตย้อนหลัง ทำการเก็บข้อมูลทางคลินิกและตัวอย่างเลือดจากผู้ป่วย 95 รายซึ่งได้รับยาอิมมาทินิบจากโรงพยาบาลรามาธิบดีระหว่างปี พ.ศ. 2544 ถึง 2560 และนำสารพันธุกรรมไปจีโนไทป์ และวิเคราะห์สมดุลฮาร์ดี-ไวน์เบิร์ก และความสัมพันธ์โดยสถิติไคว์สแควร์ ผลการศึกษาพบว่าความถี่จีโนไทป์ชนิด CC (12.63%), CT (52.63 %) และ TT (34.74%) อยู่ในสมดุลฮาร์ดี-ไวน์เบิร์ก และผู้ป่วยที่มีจีโนไทป์ชนิด CC หรือ CT มีความสัมพันธ์อย่างมีนัยสำคัญทางสถิติกับการที่มีอัตราการตอบสนองระดับอณูชนิดเมเจอร์ คือ ระดับ BCR-ABL1^{IS} น้อยกว่าหรือเท่ากับร้อยละ 0.1 หรือ มีการตอบสนองระดับอณูชนิดคอมพลีท คือ ตรวจไม่พบระดับ BCR-ABL1 transcripts ต่ำกว่าผู้ป่วยที่มีจีโนไทป์ชนิด TT (OR = 7.68, 95%CI = 0.952-61.949, p = 0.030) ผลการศึกษาชี้ให้เห็นว่า *ABCB1* C1236T ส่งผลต่อการรักษาทางคลินิกของยาอิมมาทินิบในผู้ป่วยโรคมะเร็งเม็ดเลือดขาวเรื้อรังชนิดมัยอีลอยด์ชาวไทย

คำสำคัญ: *ABCB1* C1236T, อิมมาทินิบ, โรคมะเร็งเม็ดเลือดขาวเรื้อรังชนิดมัยอีลอยด์, การตอบสนองระดับอณู

Introduction

Chronic myeloid leukemia (CML) is a type of myeloproliferative disorder. The molecular pathology of CML is the presence of *BCR-ABL1* gene resulting from the translocation of chromosome 9 and 22. The *BCR-ABL1* gene produces an abnormal BCR-ABL protein resulting in the increase of tyrosine kinase signaling pathway in hematopoietic myeloid lineage which further leads to the formation of leukemia cells. According to the European LeukemiaNet (ELN) recommendations for the management of CML, the goal of treatment is achieving major molecular response (MMR^3) and MMR^4 for treatment free remission (TFR) candidates (1). Unfortunately, some CML patients failed to achieve a major molecular response.

Imatinib was approved by the Food and Drug Administration of the United States in 2002 as the first tyrosine kinase inhibitor (TKI) for CML treatment. In Thailand, the government issued imatinib under the National List of Essential Medicines (NLEM) in 2008 for patients with CML (2). However, there have been some CML patients failing to achieve the clinical outcomes from imatinib treatment. Imatinib has been reported to be a substrate of ABC drug efflux transporter or p-glycoprotein (P-gp) specifically encoded by *ABCB1* gene (3). Apart from its constitutive expression in various tissues including the intestines, liver, kidney, adrenal gland, placenta, testis and blood-brain barrier, P-gp expression is also found in lymphocytes, hematopoietic stem cells and cancer cells. The overexpression of P-gp may limit its substrate availability at their intracellular site of action and lead to drug resistance. The *ABCB1* gene that encodes P-gp is highly polymorphic. Several studies suggest that polymorphisms in *ABCB1* gene may influence the expression or function of P-gp (4, 5). Among various single nucleotide polymorphisms (SNPs), C1236T, C3435T and G2677T/A of *ABCB1*, located in exons 12, 21 and 26, respectively, are the most widely studied SNPs. Interestingly, the study from Agrawal et al. revealed that variants of C1236T was associated with higher ABCB1 expression compared to the wild type (6).

In terms of the association of *ABCB1* polymorphisms with clinical outcomes of imatinib therapy, Ni et al. found that imatinib resistance in Chinese CML patients was associated with *ABCB1* 1236 TT genotype compared to the CT/CT genotyped group (7). In line with that, Deenik et al. suggested that variants in *ABCB1* C1236T were associated with the decrease in molecular response in Caucasian CML patients (8). While Dulucq et al. found that the variants allele was associated with imatinib response in Caucasian CML patients (9). Because of these inconclusive findings, the aim of this study was to determine the association of *ABCB1* C1236T with clinical outcomes of imatinib in Thai CML patients.

Materials and Methods

Patients

This study used an observational retrospective design. Ninety-five Thai patients from Ramathibodi Hospital between 2001 and 2017 were studied. The inclusion criteria were Thai patients over 15 years old who were diagnosed with CML and treated with imatinib for at least 3 months. The exclusion criteria were patients with mutations in *BCR-ABL1* fusion gene related to the failure to imatinib therapy, a progression from chronic stage to accelerated or blast phase, non-compliance to treatment, an unclear history of treatment or unidentified imatinib responsive status, pregnancy/breast-feeding while receiving imatinib therapy, or an alcohol/drug addiction history. An approval of the protocol (Identification number 04-59-27)

was obtained from the institutional review board of the Faculty of Medicine, Ramathibodi Hospital. Written informed consents were obtained from all participants.

Clinical assessment

In terms of clinical outcomes, molecular response outcome was defined according to the European LeukemiaNet recommendations for the management of CML. Molecular response was presented by the *BCR-ABL1* mRNA transcription ratio which was evaluated by the real time quantitative polymerase chain reaction (RQ-PCR) according to the International Scale (IS) as a ratio of BCR-ABL1% on a log scale. Major molecular response (MMR) and Complete molecular response (CMR) were defined as a ratio of BCR-ABL1^{IS} $\leq$ 0.1% and an undetected *BCR-ABL1* mRNA transcription ratio, respectively (1). Therefore, in this study CML patients were divided into two groups specifically those with BCR-ABL1^{IS} $\leq$ 0.1% or MMR/CMR and those with BCR-ABL1^{IS} > 0.1% or non-MMR/CMR.

DNA isolation and Genotyping

Peripheral blood samples were collected in EDTA tubes from all participants. DNA samples were extracted from leukocytes by using QIAamp Blood Kit (Qiagen, Hilden, Germany). The genotyping of *ABCB1* C1236T was performed by using TaqMan® SNPs genotyping assay (Thermo Fisher Scientific, USA).

Statistical Analysis

Patient characteristics and clinical data were presented as median (range). and frequency with percentage. Differences between MMR/CMR and non-MMR/CMR groups were compared using either Mann–Whitney U test or Chi-square test, as appropriate. Hardy–Weinberg equilibrium and genetic association of *ABCB1* C1236T with clinical outcomes were determined by Chi-square test. The results were reported as odds ratio and 95% confidence interval (95% CI). Statistical significance for all tests was set at a p-value < 0.05. Statistical analyses were performed using SPSS version 22.0 software (SPSS, Chicago, IL, USA).

Results

Patient Characteristics

Demographic and clinical characteristics of 95 Thai CML patients who were treated with imatinib for at least 3 months in this study are shown in Table 1. In terms of clinical outcomes, there were 82 patients with BCR-ABL1^{IS} $\leq$ 0.1% or MMR/CMR group and 13 patients with BCR-ABL1^{IS} > 0.1% or non-MMR/CMR group. No differences in demographic and clinical data between the two groups except for the duration of imatinib usage were found. Patients with MMR/CMR were more likely to be treated for more than 1 year (p < 0.001).

For prevalence of *ABCB1* C1236T, the allelic and genotype frequencies in Thai patients with CML are shown in Table 2. All genotype frequencies were in accordance with Hardy-Weinberg equilibrium (p > 0.05). The allele frequencies of C and T alleles were 38.95% and 61.05%, respectively.

Regarding the association of *ABCB1* C1236T with molecular response to imatinib in Thai patients with CML, genotype of CC or TT was significantly associated with non-MMR/CMR when compared to those with TT genotype (OR = 7.68, 95%CI = 0.952-61.949, p = 0.030) as shown in Table 3.

Table 1. Demographic and clinical characteristics of 95 Thai patients with CML

Characteristics	MMR/CMR (BCR-ABL1^{IS} ≤ 0.1%), n (%)	non-MMR/CMR (BCR-ABL1^{IS} > 0.1%), n (%)	*p*-value
Body weight (kg), median (range)	63.25 (40.30-101.10)	57.20 (45.90-95.00)	0.475[b]
Age at the start of imatinib (years), median (range)	42 (15-72)	47 (13-60)	0.965[b]
Duration of imatinib usage (years)			
> 1	80 (97.56)	3 (23.08)	<0.001[a]
≤ 1	2 (2.44)	10 (76.92)	
Diagnostic phase			
Chronic phase	68 (82.93)	12 (92.31)	0.389[a]
Accelerated phase	14 (17.07)	1 (7.69)	
Imatinib dose (mg/day)			
300	6 (7.32)	1 (7.69)	0.099[a]
400	62 (75.61)	7 (53.85)	
600	10 (12.19)	5 (38.46)	
800	4 (4.88)	0 (0)	
Gender			
Female	38 (46.34)	7 (53.85)	0.615[a]
Male	44 (53.66)	6 (46.15)	

[a] *p*-value was determined by Pearson Chi-square test.
[b] *p*-value was determined by Mann–Whitney U test.

Table 2. Genotype distributions and allele frequencies of *ABCB1* C1236T in 95 Thai patients with CML

ABCB1 C1236T Genotype	n (%)	Allele Frequency (%)	Hardy-Weinberg equilibrium *p*-value
Homozygous wild type (CC)	12 (12.63)	C = 38.95	0.2983 [a]
Heterozygous (CT)	50 (52.63)	T = 61.05	
Homozygous variant (TT)	33 (34.74)		

[a] *p*-value was determined by Pearson's Chi-square test.

Table 3. Association of *ABCB1* C1236T with molecular response to imatinib in Thai patients with CML

ABCB1 C1236T Genotype	n (%)	MMR/CMR (BCR-ABL1^{IS} ≤0.1%), n (%)	non-MMR/CMR (BCR-ABL1^{IS} >0.1%), n (%)	*p*-value	OR	95%CI
CC+CT	62 (75.26)	50 (61.00)	12 (92.30)	0.030[a]	7.68	0.952-61.949
TT	33 (34.74)	32 (39.00)	1 (7.70)			

[a] *p*-value was determined by Fisher's exact test (two-tailed).

Discussions

Genetic and non-genetic factors may be responsible for the inter-individual variability in drug response. In this study, we found no differences in demographic and clinical characteristics between the MMR/CMR and non-MMR/CMR groups except for the duration of imatinib usage. This duration of treatment was a surrogate for the difference in response to imatinib treatment. Almost patients with BCR-ABL1^{IS} >0.1% stopped imatinib treatment and switched to second- and third- line drug therapy. Whereas, patients achieving MMR/CMR were treated continuously with imatinib.

Interestingly, the present study showed the association of *ABCB1* C1236T with molecular response of imatinib in Thai patients with CML. Patients who carried CC or CT genotype of *ABCB1* C1236T were more likely to have BCR-ABL1^{IS} > 0.1%. The study by Dulucq et al. also found that *ABCB1* 1236T allele was associated with major molecular response (MMR) while the 1236C allele was associated with resistance (9). However, the effect of C1236T on the clinical outcomes of imatinib are still inconclusive. There are some studies reporting that the TT genotype of *ABCB1* 1236 was associated with imatinib resistance (7, 8).

The inconsistency of association studies may be resulted from the difference of allele and genotype frequency in each ethnicity. The distribution of allele frequency of *ABCB1* C1236T found in this study was consistent with those of other Asian populations (7, 10, 11) but different from that in Caucasians (9). Difference in genetic variant distribution among ethnicities may lead to the variation in phenotypes and response outcomes.

In addition, the incongruence of phenotype definition in each study was considered. The definitions of “response” or “resistance” in the above-mentioned studies were varied. According to the ELN recommendation, three types of response are defined including hematologic, cytogenetic, and molecular responses. Moreover, levels of response in each type were further categorized according to clinical parameters. In this study, molecular response was categorized into MMR/CMR based on the *BCR-ABL1* mRNA transcription ratio. Therefore, the difference in clinical outcomes among various studies may result in different findings. Furthermore, the term “resistance” was incongruently defined at the time of monitoring. Resistance to imatinib can be defined either as the primary resistance (a lack of efficacy from the time of treatment with imatinib) or the secondary resistance (relapse or recurrence) (1). Since all patients with BCR-ABL1^{IS} > 0.1% were failed to achieve MMR in this present study, nevertheless, the number of patients in this group was lower than BCR-ABL1^{IS} ≤ 0.1%. Therefore, patients who carried CC/CT genotypes of *ABCB1* C1236T were more likely to face primary resistance of imatinib when compared to those with TT genotype.

In term of genetic variation, some evidences suggested that variants in the gene encoding P-gp may affect therapeutic outcomes of their substrate. Hoffmeyer et al. first reported that *ABCB1* C3435T, a synonymous SNP, was associated with P-gp expression in the small intestine. They found that the TT genotype had a P-gp expression lower than that of CC genotype and associated with a higher plasma digoxin concentration (12). Owing to the Linkage disequilibrium (LD) in the three SNPs namely C1236T, C3435T and G2677T/A (13), Kimchi-Sarfaty et al. revealed that the TTT haplotype of these SNPs might influence the conformational changes including specific binding site of P-gp without alteration of mRNA protein levels. These alterations may differently affect interaction sites depending on its substrate leading to the lack of efficiency outcomes (14).

Conclusion

This study evaluated the association of polymorphisms in *ABCB1* with therapeutic outcomes of imatinib in Thai patients with chronic myeloid leukemia. The finding revealed that *ABCB1* C1236T influenced the molecular response to imatinib. Further study is needed to determine the association of other transporter genes, such as *OCT*, *ABCG2* and genes encoding imatinib metabolizing enzymes such as *CYP3A4/3A5*, with the treatment outcomes of imatinib in a larger sample size of patients with CML.

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B11 Effect of *CYP2C19* Polymorphisms on Voriconazole Dosage Regimen in Thai Invasive Fungal Infection Patients

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Abstract

Voriconazole is an antifungal agent recommended as the first line treatment for invasive aspergillosis infection. Voriconazole has a narrow therapeutic range and zero order metabolism. Therefore, therapeutic drug monitoring needs to be performed to achieve a therapeutic level (1-5 µg/ml). The previous study reported *CYP2C19* polymorphisms correlated with the voriconazole trough concentration. The aim of this study was to determine correlation between the voriconazole dosage regimen at therapeutic range and *CYP2C19* polymorphisms in Thai invasive fungal infection patients. Moreover, prevalence of *CYP2C19* allelic variants in Thai healthy volunteers was investigated comparing that to other ethnic groups. One hundred Thai healthy volunteers and 80 invasive fungal infection patients treated with voriconazole were enrolled. *CYP2C19* polymorphisms were genotyped in both healthy volunteers and the patients. The prevalence of Thai *CYP2C19* variants (*CYP2C19*1/*1* 50%, **1/*2* 36%, **1/*3* 6%, **2/*2* 6%, **2/*3* 1% and **1/*17* 1%, respectively) was similar to Korean and Chinese populations, but it was different from Japanese, Caucasian, Ugandan and Russian populations. Moreover, correlation between the voriconazole dosage regimen and the *CYP2C19* genotypes was determined. The patients with poor metabolizers tended to require lower dose of voriconazole than patients with extensive and intermediate metabolizers (7.31±0.72 VS 8.38±0.31 mg/day, respectively, P=0.298). Nevertheless, no statistically significant difference of the correlation between *CYP2C19* mutations and voriconazole doses was found in this study.

Keywords: *CYP2C19* polymorphisms, Voriconazole, Dosage regimen, Invasive fungal infection

ผลของความผิดแผกทางพันธุกรรมของยีน *CYP2C19* ต่อขนาดยาวอริโคนาโซลในผู้ป่วยติดเชื้อราลุกลามชาวไทย

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บทคัดย่อ

ยาวอริโคนาโซลเป็นยาต้านเชื้อราลำดับแรกที่ถูกนำมาใช้รักษาผู้ป่วยติดเชื้อราลุกลามชนิดแอสเปอร์จิลัส ยาวอริโคนาโซลนี้มีช่วงการรักษาที่แคบและมีรูปแบบการเมแทบอลิซึมเป็นแบบ zero order ดังนั้นจึงต้องมีการติดตามการรักษาด้วยการเจาะระดับยาในเลือด (therapeutic drug monitoring) เพื่อให้ความเข้มข้นของยาวอริโคนาโซลอยู่ในระดับรักษา (1-5 μg/ml) จากการศึกษาก่อนหน้าพบว่า ความผิดแผกทางพันธุกรรมของยีน *CYP2C19* มีความสัมพันธ์กับระดับยาวอริโคนาโซล ดังนั้นในการศึกษานี้จึงมีวัตถุประสงค์เพื่อศึกษาความสัมพันธ์ของความผิดแผกทางพันธุกรรมของยีน *CYP2C19* กับขนาดยาวอริโคนาโซลที่ผู้ป่วยติดเชื้อราลุกลามควรได้รับเมื่อระดับยาเข้าถึงระดับรักษา และศึกษาความชุกของ *CYP2C19* ในอาสาสมัครชาวไทยสุขภาพดี โดยอาสาสมัครสุขภาพดีทั้งหมด 100 คน ถูกเก็บเลือดและตรวจหาจีโนไทป์ของ *CYP2C19* ในขณะที่ผู้ป่วย 80 คนที่เคยเข้ารับการรักษาการติดเชื้อลุกลาม และได้รับยาวอริโคนาโซล ถูกเก็บข้อมูลทางคลินิก ผลการศึกษาในอาสาสมัครสุขภาพดีพบว่า การเกิดมิวเตชั่นของ *CYP2C19* ในประชากรชาวไทย (*CYP2C19*1/*1* 50%, **1/*2* 36%, **1/*3* 6%, **2/*2* 6%, **2/*3* 1% และ **1/*17* 1%, ตามลำดับ) มีความถี่ใกล้เคียงกับประชากรชาวเกาหลีและจีน แต่แตกต่างกับประชากรญี่ปุ่น คอเคเชี่ยน อูกันดา และรัสเซีย นอกจากนี้ยังพบว่าผู้ป่วยที่มีการทำงานของเอนไซม์ CYP2C19 กลุ่ม poor metabolizer มีแนวโน้มที่ต้องการยาวอริโคนาโซลในขนาดต่ำกว่าผู้ป่วยที่มีการทำงานของเอนไซม์ดังกล่าวปกติ (7.31±0.72 VS 8.38±0.31 mg/day, respectively, P=0.298) อย่างไรก็ตามยังไม่พบความสัมพันธ์ของความผิดแผกทางพันธุกรรมกับขนาดยาอย่างมีนัยสำคัญ

คำสำคัญ: ความผิดแผกทางพันธุกรรมของยีน CYP2C19, ยาวอริโคนาโซล, การติดเชื้อราลุกลาม

Introduction

Invasive fungal infections (IFIs) are a major cause of mortality in immunocompromised patients such as those receiving chemotherapy or haematopoeitic stem cell transplant (HSCT). Invasive aspergillosis infection was found as the most infectious disease in these patients (43%) (1). Moreover, the attributable mortality rate of aspergillosis has increased to 72.1% (2). Similarly, the common invasive fungal infections in Thai immunocompromised patients were *Candida spp.* and *Aspergillus spp.* (3). Voriconazole is recommended as a first line drug for invasive aspergillosis treatment.

Voriconazole is a second-generation of triazole antifungal agent. It was used as the first line IFIs therapy because its broad-spectrum activity can be against both yeasts and molds including fluconazole resistant candida, *Scedosporium apiospermum* and *Fusarium spp.* It is administered in three forms: intravenous, oral tablet and suspension (4). The dose of intravenous route is suggested at 6 mg/kg every 12 hours for 2 doses for loading dose, followed by 4 mg/kg every 12 hours for maintenance dose. An oral dose of voriconazole of 400 mg twice a day is prescribed as a loading dose the first day, followed by 200 mg twice daily (5). However, voriconazole performs nonlinear pharmacokinetic profiles with a narrow therapeutic concentration, 1-5 µg/ml. Therefore, inter- and intra-individual variabilities that may lead to toxicity or decreased efficacy should be considered. Voriconazole levels need to be closely monitored to improve efficacy and safety of IFIs treatment (6).

Voriconazole metabolism primarily occurs in liver by cytochrome P450 isoenzymes (CYP), primarily by CYP2C19 and to some small extent by CYP3A4 and CYP2C9. Many studies reported *CYP2C19* genetic variation correlated with the voriconazole exposure. The voriconazole trough concentrations of extensive metabolizers (EM, *CYP2C19*1/1*) patients were lower than intermediate metabolizers (IM, *CYP2C19*1/2, *1/*3*) and poor metabolizing enzymes (PM, *CYP2C19*2/2, *2/*3, *3/*3*) patients. Conversely, ultrarapid metabolizing patients (URM, *CYP2C19*17*) had significant lower voriconazole concentrations than EM group (4, 7). Therefore, the URM group may require the higher voriconazole dosage regimen (3.94±0.39 for *CYP2C19*1/*17* and 6.75±0.54 for *CYP2C19*17*17* VS 2.57±0.25 mg/kg twice daily for wild type; $P<0.0001$), while PM carriers may receive the lower dose than EM carriers to achieve the same therapeutic range (3.03±0.36 VS 2.57±0.25 mg/kg twice daily) (7). A multicenter study of voriconazole showed voriconazole concentration <1.7 mg/l was correlated with a significant incidence of treatment failure than voriconazole concentration ≥1.7 mg/l (26% VS 7%, $P<0.01$). Neurotoxicity, such as auditory and visual hallucinations, occurred more frequently at voriconazole blood level > 5 mg/l than at voriconazole blood level ≤ 5 mg/l (32% VS 1.2%, $P<0.01$) (8). Therefore, receiving the voriconazole dose based on individually guided genotypes may allow the patients to achieve therapeutic range faster than conventional treatment. Moreover, genotype guided regimen may decrease adverse effects in PM and increase efficacy in URM patients. These may improve the clinical outcome and prognosis of IFI patients treated with voriconazole.

This study aimed to determine the correlation between the voriconazole dosage regimen at therapeutic range and *CYP2C19* polymorphisms in Thai invasive fungal infection patients. Moreover, the prevalence of *CYP2C19* allelic variants in Thai healthy volunteers was analyzed comparing to other ethnic groups.

Material and Methods

Subjects

This was a retrospective study. Eighty patients were enrolled in this study. The study was approved by the Ethics Committee, Ramathibodi Hospital, Mahidol University. The clinical data of the patients was recorded including voriconazole trough concentration, voriconazole dosage regimen and *CYP2C19* genotypes. Moreover, 100 non-related Thai healthy volunteers who were blood donors at Blood Bank of Faculty of Medicine, Khon Kaen University were enrolled to determine frequencies of *CYP2C19* variants related clinical variables such as *CYP2C19*2* (rs4244285; 681G>A), *CYP2C19*3* (rs4986893; 636G>A) and *CYP2C19*17* (rs12248560; -806C>T). This study was carried out based on the Declaration of Helsinki and the ICH Good Clinical Practice Guidelines approved by the Ethics Committee, Khon Kaen University.

DNA extraction and CYP2C19 genotyping

Genomic DNA (gDNA) of the patients had been already extracted by automated MagNA Pure Compact machine (Roche Applied Science, Penzberg, Germany) based on magnetic-bead technology. The same as *CYP2C19* genotyping, they have been already detected by LightMix®. It analyzed the variants based on a principle of Real-time PCR. The healthy participants' blood was collected in an EDTA tube and was extracted by QIAamp® DNA blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction. The variant alleles were genotyped by Light-Cycler 480® based on Real-time PCR technique with specific TaqMan® probe and primer following the previous study (4).

Statistical analysis

Allele frequencies of *CYP2C19* polymorphisms were calculated and compared with other ethnic groups using Pearson Chi-square test or Fisher's exact test. The correlation between *CYP2C19* variants and the voriconazole dose were measured by One-way Anova. All statistical tests were two-tailed, and p-value less than 0.05 was expected as statistically significant. SPSS (Statistical Package for the Social Science for Windows, version 19.0, IBM Corp., New York) was used for all analysis.

Results

Phenotype frequencies of CYP2C19 polymorphisms in different ethnics

CYP2C19 genotype was evaluated in 100 Thai healthy volunteers. The frequencies were categorized in form of the phenotype frequencies in Table 1. Prevalence of EM, IM, PM and URM in Thai healthy volunteers were 50%, 42%, 7% and 1%, respectively. These frequencies were not significantly different from previous Thai prevalence studies, Korean and Chinese population (Table 1) (9, 10). However, there was a significant difference from Japanese, Caucasian, Ugandan and Russian populations (9, 11-13).

Table 1. The frequencies of CYP2C19 phenotype in different ethnics

Ethnic group	N	CYP2C19 phenotype frequencies (%)				*p-value*	Ref.
		EM (1/*1*)**	**IM (**1/*2, *1/*3*)**	**PM (**2/*2, *2/*3*)**	**URM (**1/*17, *17/*17*)**		
Thai	100	50 (50.0)	42 (42.0)	7 (7.0)	1 (1.0)		This study
Thai (previous study)	1,051	428 (40.72)	441 (41.96)	137 (13.04)	45 (4.28)	**0.072**	Sukasem C, 2013
Japanese	261	94 (36.0)	116 (44.4)	48 (18.4)	3 (1.1)	**0.012**	Sugimoto K, 2007
Korean	100	40 (40.00)	48 (48.0)	9 (9.0)	3 (3.0)	**0.433**	Myrand SP, 2008
Chinese	100	43 (43.0)	47 (47.0)	9 (9.0)	1 (1.0)	**0.838**	
Caucasian	133	57 (42.86)	27 (20.3)	2 (1.50)	47 (35.34)	**< 0.001**	
Ugandan	90	50 (55.56)	14 (15.55)	2 (2.22)	24 (26.67)	**< 0.001**	Miura J, 2008
Russian	892	317 (35.54)	172 (19.28)	17 (1.91)	386 (43.27)	**< 0.001**	Sychev D, 2015

Data in parentheses represent percentage of each phenotype.

Demographic data and clinical characteristics

Demographic and clinical data of 80 patients were collected and analyzed (table 2). Forty-three patients (53.75%) were female. The mean of age and body weight was 54.81 years and 56.15 kg, respectively. Most patients were diagnosed as haematological malignancy (45/80, 56.25%) and the most common infected site was the lungs (57/80, 71.25%). Eighty-five percent of patients received oral voriconazole at steady state.

Table 2. Demographic data and clinical characteristics of the study subjects (N = 80)

Demographic characteristics	Category	Value of characteristics (%)
Sex	Male	37 (46.25)
	Female	43 (53.75)
Age (year), range		54.81 ± 17.82 (18-87)
Body weight (kg), range		56.15 ± 10.79 (34-88)
Underlying disease	Haematological malignancy	45 (56.25)
	Haematological disease	3 (3.75)
	Kidney disease	11 (13.75)
	Lung disease	4 (5.00)
	SLE	3 (3.75)
	Other	14 (17.50)
Site of infection	Pulmonary	57 (71.25)
	Extra pulmonary such as sinus, eyes and brain	23 (28.75)
Route of administration at steady state	Intravenous	12 (15)
	Oral	68 (85)

The prevalence of CYP2C19 genotype and voriconazole dosage regimen in IFI patients

Genotype of *CYP2C19* polymorphisms in IFI patients were analyzed and the correlation between phenotype and voriconazole dosage regimen required to achieve therapeutic range was evaluated (Table 3). Phenotype frequencies of CYP2C19 were EM 43.75%, IM 41.25% and PM 15%. Voriconazole doses required to achieve therapeutic range for EM, IM, and PM were 8.38 ± 0.31, 8.19 ± 0.34 and 7.31 ± 0.72 mg/kg/day, respectively. However, the mean of voriconazole doses was not significantly statistically different related to the CYP2C19 phenotypes.

Table 3. Voriconazole dosage regimen with CYP2C19 phenotypes

***CYP2C19* polymorphisms**		**Number of patients (%) n = 80**	**Mean of voriconazole dose (mg/kg/day)**	**P-value[a]**
EM	***1/*1**	35 (43.75)	8.38 ± 0.31	**0.298**
IM	***1/*2**	29 (36.25)	8.19 ± 0.34	
	***1/*3**	4 (5.00)		
PM	***2/*2**	8 (10.00)	7.31 ± 0.72	
	***2/*3**	4 (5.00)		

a: comparison within three groups of the phenotype by One-way Anova

Discussion

CYP2C19 is the main metabolic enzyme responsible for voriconazole metabolism. Recent data showed *CYP2C19* genetic polymorphism affected voriconazole clearance. Therefore, personalized voriconazole dosage regimen to achieve the same therapeutic dose depended on *CYP2C19* genotype of the patients. *CYP2C19* genotypes varied between ethnic groups. Therefore, this study aimed to investigate the correlation between *CYP2C19* variants and voriconazole dose in Thai IFI patients and prevalence of *CYP2C19* genotype in Thai healthy volunteer. The phenotype frequencies in Thais were not different from Asian populations except Japanese because of 2.6 times lower prevalence of PM in Thais than Japanese. This was similar to Tassaneeyakul's study which reported the prevalence of PMs in North-Eastern Thai healthy population was significantly lower than Japanese populations (14). The most Thai healthy volunteers in our study were in North-Eastern region of Thailand, as Tassaneeyakul's study. In contrast, Sukasem's study did not well define the region of the patients. His study showed the prevalence of the PM patients was consistent with other Asian population studies including Japanese (10). Therefore, this study might confirm the hypothesis that "The heterogeneity among Thai population of *CYP2C19* polymorphism should be recognized" (15). Moreover, prevalence of *CYP2C19* polymorphisms in this study was different from Caucasian and African populations. These populations had higher frequencies of *CYP2C19*17* allele than Asian populations.

Previous studies showed CYP2C19 phenotypes correlated with voriconazole trough concentrations (4, 7, 16). EM patients may require 15.18% higher dose of voriconazole than PM and IM patients, while EM carriers required a significant 61.93% lower dose than URM group (7). In our study, voriconazole dose in EM patients is 14.64% higher than PM.

However, no significant correlation between CYP2C19 phenotype and voriconazole dose were observed because of small population and lack of *CYP2C19*17* allele.

There were a few limitations of this study. First, this study was a retrospective observational study. Second, there was the limited number of patients (n = 80) leading to a limited number of PM and URM groups. Further study should prospectively examine this correlation between voriconazole dose and *CYP2C19* genotype and investigate other genetic effects on voriconazole metabolism such as *CYP3A4* or *FMOs*.

Conclusion

The prevalence of CYP2C19 phenotype in Thais was similar to other Asian populations except Japanese. But it was different from Caucasian and African populations. However, the sample size of IFI patients was too low to determine the correlation between *CYP2C19* polymorphisms and voriconazole dosage regimen.

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B12 Effect of Candidone, a Compound from *Derris Indica*, on Apoptosis Induction and Cell Migration Suppression in Cholangiocarcinoma Cell Line KKU-M156

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Abstract

Cholangiocarcinoma (CCA) is a fatal malignancy that arising from biliary epithelium. To date, there is no curative treatment option except surgery. However, most of CCA patients are in inoperable stage. Thus, effective anticancer agents are urgently needed to improve treatment outcome for CCA patients. Candidone (CD) is a flavanone compound from *Derris indica*, exhibits anticancer activity against various cancer cells. Here, we investigated the antitumor effects of CD in CCA cells. CD showed potent cytotoxicity on KKU-M156 cells with the IC_{50} of 4.24 µg/mL at 24 h. It significantly inhibited cell migration and induced apoptosis. In addition, CD exerted antitumor effect through upregulation of anti-proliferative and pro-apoptotic genes, *p21* and *Bax,* respectively. Our findings suggest that CD could be a potential anticancer agent for CCA treatment.

Keywords: Candidone, *Derris indica*, cholangiocarcinoma, cell migration, apoptosis

ผลของสารแคนดิโดนที่สกัดจากต้นหยีน้ำ (*Derris indica*) ต่อการเหนี่ยวนำการตายแบบอะพอพโทซิสและยับยั้งการเคลื่อนที่ของเซลล์มะเร็งท่อน้ำดี

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บทคัดย่อ

มะเร็งท่อน้ำดี เป็นมะเร็งชนิดร้ายแรงถึงชีวิตซึ่งเกิดมาจากเยื่อบุผนังทางเดินน้ำดี ในปัจจุบันนี้ ยังไม่มีแนวทางการรักษามะเร็งท่อน้ำดีด้วยวิธีใดที่มีประสิทธิภาพยกเว้นวิธีการรักษาด้วยการผ่าตัด อย่างไรก็ตาม ผู้ป่วยโรคมะเร็งท่อน้ำดีส่วนใหญ่อยู่ในระยะมะเร็งลุกลาม ส่งผลให้ผู้ป่วยในระยะนี้ไม่สามารถทำการรักษาด้วยวิธีการผ่าตัดได้ ดังนั้น การวิจัยเพื่อหาสารที่มีประสิทธิภาพต่อฤทธิ์ต้านมะเร็งจึงมีความจำเป็นอย่างยิ่งเพื่อเพิ่มผลการรักษาสำหรับผู้ป่วยมะเร็งท่อน้ำดี แคนดิโดนจัดเป็นสารประกอบกลุ่มฟลาโวนอยด์ที่สกัดได้จากต้นหยีน้ำ พบว่า แคนดิโดนมีความเป็นพิษต่อเซลล์มะเร็งหลายชนิด ในงานวิจัยนี้ทำการศึกษาฤทธิ์ต้านมะเร็งของแคนดิโดนต่อเซลล์มะเร็งท่อน้ำดี พบว่า แคนดิโดนมีความเป็นพิษสูงต่อเซลล์เซลล์มะเร็งท่อน้ำดีของมนุษย์ชนิด KKU-M156 โดยมีค่าความเข้มข้นที่ยับยั้งการมีชีวิตรอดของเซลล์ได้ 50% (IC_{50}) เท่ากับ 4.24 µg/mL ที่เวลา 24 ชั่วโมง แคนดิโดนสามารถยับยั้งการเคลื่อนที่ของเซลล์และชักนำให้เซลล์มะเร็งท่อน้ำดีเกิดการตายแบบอะพอพโทซิสอย่างมีนัยสำคัญ นอกจากนี้ แคนดิโดนมีฤทธิ์ต้านมะเร็งโดยเพิ่มการแสดงออกของยีนยับยั้งการเพิ่มจำนวนและยีนกระตุ้นการตายแบบอะพอพโทซิส ได้แก่ *p21* และ *Bax* ตามลำดับ ผลการทดลองครั้งนี้แสดงว่าแคนดิโดนมีศักยภาพในการพัฒนาเป็นยารักษามะเร็งท่อน้ำดี

คำสำคัญ: แคนดิโดน, ต้นหยีน้ำ (*Derris indica*), มะเร็งท่อน้ำดี, การเคลื่อนที่ของเซลล์, การตายแบบอะพอพโทซิส

Introduction

Cholangiocarcinoma (CCA) is biliary cancer arising from epithelial lining within the biliary tree. The classification based on anatomical location includes intrahepatic, perihilar, and distal CCA (1). The incidence and mortality rates are increasing worldwide. In Thailand, the highest incidence has been reported in the Northeastern region. In this area, the incidence of CCA is strongly correlated with the prevalence of liver fluke infection caused by *Opisthorchis viverrini* (2). Carcinogenesis in CCA includes alterations in the stroma, recruitment of fibroblasts, remodeling of the extracellular matrix (ECM), changing patterns of immune cell migration, and promotion of angiogenesis. The treatment of CCA for non-metastatic stage is the surgical removal of the tumor. In contrast, treatment options are limited and overall survival rates are low in metastatic CCA. Moreover, CCA has low response rate to chemotherapeutic agents including gemcitabine, cisplatin and 5-fluorouracil (3). Searching for effective compounds to be used for treatment of CCA is urgently needed.

Candidone (CD) is a flavonoid derived from the crude hexane extract of the fruit of *Derris indica* (4). This plant is widely found in the Southern Thailand. The plant has been used in traditional medicine for bronchitis, whooping cough, rheumatic joints and diabetes (5). Candidone has been shown to exhibit biological activities such as antiprotozoal, cytotoxicity against L-6, RAW and HT-29 cell lines (6). Moreover, the previous study showed that candidone had potent cytotoxicity against CCA cells (4). This study aimed to evaluate antitumor effects of candidone on proliferation, apoptosis, and migration in CCA cells.

Materials and Methods

Chemical and reagent

Candidone was provided by Assoc.Prof. Chavi Yenjai, Natural Products Research Unit, Department of Chemistry and Centre for Innovation in Chemistry, Faculty of Science, Khon Kaen University. Candidone is a pure compound from the hexane extracted from fruits of *Derris indica* as described in the previous study (4). Sulphorhodamine B (SRB), trichloroacetic acid, acridine orange, ethidium bromide and TRIzol reagent were obtained from Sigma-Aldrich (MO, USA). Insulin like growth factor -1 (IGF-1) human recombinant was purchased from Prospec-Tany Technogene Ltd. (NJ, USA). The reagents for RT-qPCR such as iScript™ cDNA Synthesis Kit, TaqMan™ Fast Advanced Master Mix and Taqman probes (*CDKN1A*, Hs00355782_m1, *Bax*, Hs00180269_m1 and β-actin, Hs99999903_m1) were purchased from Biorad (CA, USA) and Applied Biosystems™ (CA, USA), respectively.

Cell line and cell culture

A human CCA cell line derived from poorly differentiated adenocarcinoma obtained from patient's sample, KKU-M156 was kindly provided by Prof. Banchob Sripa, Department of Pathology, Faculty of Medicine, Khon Kaen University. Cells were cultured in Ham's F12 medium supplemented with 100 U/mL penicillin, 100 µg/mL gentamycin sulphate and 10% fetal bovine serum. The cells were maintained under 5% CO_2 atmosphere at 37 °C and were subcultured every 3 days using 0.25 % trypsin–EDTA.

Cytotoxicity assay

The cytotoxicity of CD on CCA cells was examined by the SRB assay. Briefly, KKU-M156 cells were seeded in 96-well plates at the density of 7,500 cells/well in 100 µL of Ham's F12 media and allowed to grow overnight. Next day, the cells were treated with CD (0-10 µg/mL) for 24 h. After an incubation period, cells were fixed with ice-cold 10% trichloroacetic acid for 1 h at 4 °C, and subsequently stained with 0.4% SRB in 1% acetic acid for 30 minutes. The protein-bound dye was dissolved with 10 mM Tris base solution 200 µL for determination of absorbance with microplate reader with a filter wavelength of 540 nm.

Wound healing assay

KKU-M156 cells were seeded at density 250,000 cells/well into a 24-well plate and allowed to grow overnight. Next day, a scratched wound was made with a sterile 200 µL pipette tip. After washed with sterilized PBS to remove any detached cell, the culture medium was removed then cells were treated with insulin like growth factor-1 (IGF-1) 50 nM alone or with various concentrations of CD. A series of images of the scratched wound were taken from 0-48 h. The closing of scratched wound represented the cell migration, were analyzed by Image-Pro Plus software (Media Cybernetics, LP, USA).

Acridine orange/ Ethidium bromide (AO/EB) staining

KKU-M156 cells were seeded into 96-well culture plates at the density of 7,500 cells/well and allowed to attach overnight. On the next day, the medium was removed and the cells were treated with various concentrations of CD for 48 h. At the end of the treatment, cultured cells were rinsed with PBS and stained with AO and EB (each 1 µg/mL). The fluorescent images were captured using a Nikon Eclipse TS100 inverted microscope with excitation and long-pass emission filters of 480 and 535 nm, respectively. The numbers of live and apoptotic cells were counted and calculated as the percentage of cells over a total number of cells in the same area.

Quantitative real-time polymerase chain reaction

KKU-M156 cells were seeded at a density of 300,000 cells/well in 6 well-plates and allowed to grow for overnight. The cells were treated with 0 and 5 µg/mL of CD for 24 h, total RNA was extracted using TRIzol reagent according to the manufacturer's instructions. Then 1 µg total RNA was converted to cDNA using iScript™ cDNA Synthesis Kit following the manufacturer's protocol. The 50 ng of cDNA was used for a template for real-time PCR. The mRNA expression levels of *p21*, *Bax* and β-actin were analyzed with Taqman gene expression assay using TaqMan™ Fast Advanced Master Mix and Taqman probes on a QuantStudio™ 6 Flex Real-Time Refurbished PCR System (Applied Biosystems™, CA, USA). The relative expression of genes was analyzed with a cycle threshold (Ct) in the linear range of amplification. Relative mRNA expression of the interested genes was normalized to β-actin mRNA expression.

Statistical analysis

All results are presented as the mean ± SD. Statistical comparison between control and treatment group is performed using the Student's t-test and one-way analysis of variance (ANOVA). Results are considered to be statistically significant if p values were less than 0.05.

Results

Candidone suppresses cell viability in KKU-M156 cells

To determine the cytotoxicity of CD, KKU-M156 were exposed to various concentrations from 0.5-10 µg/mL for 24 h. The effect of CD on cell viability was measured by SRB assay. The results showed that CD exhibited potent cytotoxic effect on KKU-M156 cells in a dose-dependent manner with the IC_{50} of 4.24 ± 1.5 µg/mL (Figure 1).

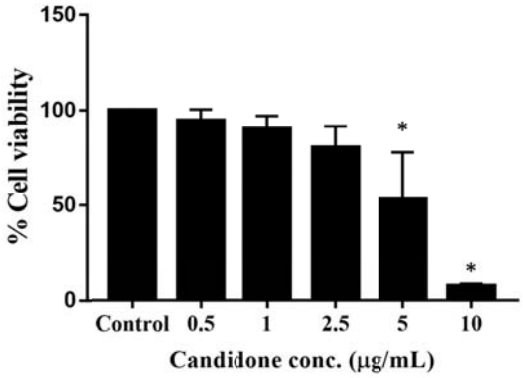

Figure 1. CD suppresses cell viability in KKU-M156 cells. The cells were treated with various concentrations of CD for 24 h. Cells were analyzed for the cell viability using SRB assay. The results are presented as the percent of cell viability. Each value represents the mean ± SD of three independent experiments. *p<0.05 compared with control.

Candidone suppresses KKU-M156 cell migration mediated by IGF-1

The effects of CD on cell migratory activity of KKU-M156 cells were examined using wound healing assay. The result showed that IGF-1 could significantly induced KKU-M156 cell migration compared to the control. Notably, CD suppressed the migratory effect mediated by IGF-1 in a dose-dependent manner after treatment for 24 h (Figure 2A and 2B) and 48 h (Figure 2A and 2C). The results clearly showed that CD significantly suppressed the IGF-1 dependent CCA cell motility.

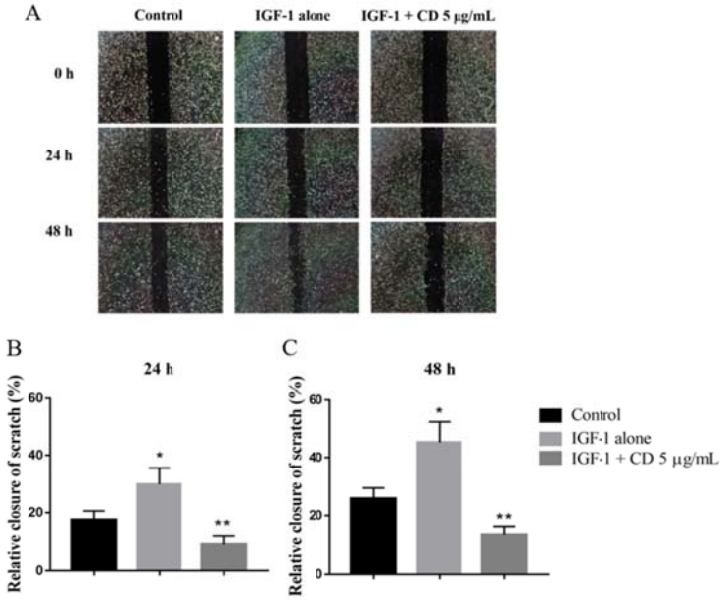

Figure 2. CD suppresses KKU-M156 cell migration mediated by IGF-1. (A) Scratched wounds of monolayer KKU-M156 cells were treated with 5 µg/mL. The representative

images from wound healing assay were taken from the same field at 0, 24 and 48 h, under phase-contrast microscopy (40X magnification). (B, C) The graph showed the percent of wound closure at 24 and 48h, representing the migratory activity of the CCA cells. The data are represented as mean ± SD each from three independent experiments. *p<0.05 compared with control. **p<0.05 compared with IGF-1 alone.

Candidone induces KKU-M156 cell apoptosis

To determine whether CD can induce apoptosis in CCA cells. KKU-M156 cells were treated with 0 and 5 µg/mL for 24 h. Cell death was assessed by fluorescence staining (AO/EB). As demonstrated in the figure 3, the levels of apoptotic cells were increased 38.3% in CD group compared to untreated control. Consistent with this result, the decrease of live cells was found in CD treated group compared to the control.

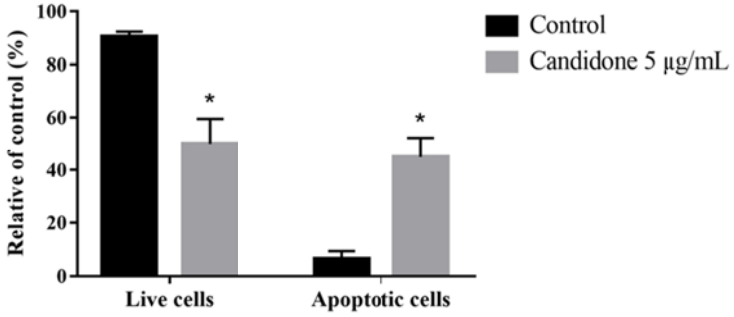

Figure 3. CD induces KKU-M156 cell apoptosis. The graph shows the relative levels of live cells and apoptotic cells compared to control. The data are represented as mean ± SD each from three independent experiments. *p<0.05 compared with control.

Effects of candidone on the expressions of genes regulating proliferation and apoptosis

To determine the underlying mechanisms by which CD suppressed cell proliferation and induced apoptosis. The expression of genes involved in regulation of cell proliferation *p21* (*CDKN1A*) and apoptosis (*Bax*) were determined using RT-qPCR. After treatment with 5 µg/mL of CD for 24 h, CD up-regulated *p21* expression compared to the control (Figure 4A). In addition, there was significantly increased in *Bax* expression compare to the untreated control (Figure 4B).

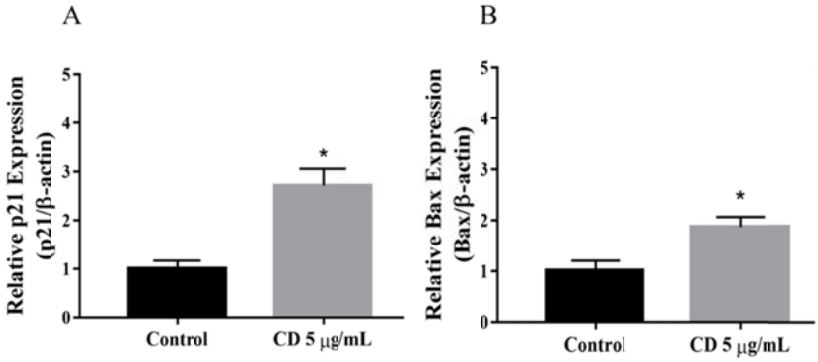

Figure 4. Effect of CD on the mRNA expression of *p21* and *Bax*. KKU-M156 cells were treated with 5 µg/mL of CD for 24 h. The reverse transcription real-time polymerase chain reaction (RT-qPCR) was carried out for *p21* and *Bax*. The expression of interested gene was normalized with β-actin mRNA expression. Data are expressed as mean ± SD each from three independent experiments. *P<0.05 compare with control.

Discussion

CCA is a biliary cancer with very poor prognosis. Most of the CCA patients present with an advanced stage after diagnosed and the curable operation is not feasible for them. The palliative treatment including systematic chemotherapy modestly prolongs survival (7). Many studies attempt to investigate the potential phytochemicals which would improve the survival outcome. Candidone is a flavanone compound from *Derris indica*. In this study, we evaluated the anticancer effect of CD on KKU-M156 cells. Our results showed that CD had cytotoxic effect against CCA cells with IC50 values in a micromolar range. The agent mediated antiproliferative effect through *p21* upregulation. *p21* is the cyclin-dependent kinase inhibitor, regulating the cell cycle as well as inhibiting cell proliferation.

Cancer cells use their intrinsic migratory ability to invade adjacent tissues and to metastasize to distant sites, result in cancer progression (8). Notably, CD strongly inhibits cell migration. Many flavonoid derivatives have been reported their antimigratory effect against various cancers. For example, genistein reduces tumor cell migration of B16FO melanoma cells (7). Luteolin also shows the activity to suppress CCA cell migration (9). Like other flavonoids, CD also exerts anticancer effect through antimigration. This result is implied that CD might be able to interrupt tumor invasion and metastasis. However, the precise mechanism is warrant to be explored in CCA.

Many phytochemicals induce apoptosis in various cancer cells such as apigenin, resveratrol and curcumin (10). The result of AO/EB assay demonstrated that CD induced apoptotic cell death in CCA. The mechanism, by which CD induced apoptosis, was associated with the upregulation of *Bax*. Proapoptotic Bax promotes apoptosis by directly regulating the activity of the Bcl-2 family proteins. Bax and Bcl-2 are co-regulators of apoptosis through the intrinsic or mitochondrial cell death pathway (11). Taken together, CD mediated antiproliferative by activating *p21* and it promoted apoptosis in CCA cells by activating proapoptotic *Bax*.

Conclusion

Candidone exhibited antitumor effects including antiproliferation, antimigration and induced apoptosis in human CCA cells by modulating the expression of genes involved proliferation and apoptosis. Thus, candidone could be potential anticancer agent to improve the treatment outcome in CCA.

Acknowledgements

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B13 Lipoxin A4 Attenuates fMLP-Induced Superoxide Anion Generation by Human Polymorphonuclear Neutrophils

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Abstract

Lipoxin A4 (LXA4) is a metabolite of the arachidonic pathway. It has been shown to act as anti-inflammatory agent by inhibiting neutrophil function such as adhesion molecule expression, neutrophil chemotaxis and transmigration. Therefore, this study was further investigated the anti-inflammatory mechanism of this agent on human neutrophil function including superoxide anion generation, myeloperoxidase production and elastase release. LXA4 was primarily investigated for neutrophil viability using XTT assay. The viability of neutrophil ($3x10^6$ cells/ml) did not affect by this compound (at the concentration of 0.1-100 µM) at a 4 hour incubation period, however a 10% reduction in neutrophil viability was observed at the concentration of 1000 µM. After incubation of human neutrophils with this compound at the concentration of 10-100 µM, superoxide anion generation induced by N-formyl-methionyl-leucyl-phenylalanine (fMLP) was inhibited. However, this compound did not affect the MPO production and elastase release induced by fMLP. The inhibitory effect of SAG on neutrophil responsiveness might be attributed, in part, to its anti-inflammatory activity.

Keywords: lipoxin A4, superoxide anion, myeloperoxidase, elastase

ไลป็อกซินเอโฟร์ลดการสร้างซุปเปอร์ออกไซด์แอนไอออนในเม็ดเลือดขาวชนิดนิวโทรฟิลจากการเหนี่ยวนำโดยสาร fMLP

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*ผู้รับผิดชอบบทความวิจัย

บทคัดย่อ

ไลป็อกซินเอโฟร์เป็นสารสื่อกลางที่ถูกสร้างขึ้นในระหว่างปฏิกิริยาการอักเสบ พบว่าสารนี้มีฤทธิ์ยับยั้งขบวนต่าง ๆ ของการอักเสบได้ เช่น ยับยั้งการแสดงออกของโมเลกุลยึดเกาะบนผิวนิวโทรฟิล รวมทั้งยับยั้งขบวนการเคลื่อนตัวของนิวโทรฟิลได้ ดังนั้นการศึกษานี้จึงได้นำสารสังเคราะห์ไลป็อกซินเอโฟร์มาศึกษาถึงกลไกต้านการอักเสบในระดับเซลล์ โดยดูผลต่อการทำงานของเม็ดเลือดขาวชนิดนิวโทรฟิล ได้แก่ การยับยั้งการสร้างซุปเปอร์ออกไซด์แอนไอออน การยับยั้งการสร้างเอนไซม์ไมอีโลเพอรอกซีเดสและการยับยั้งการหลั่งเอนไซม์อิลาสเทสในเซลล์เม็ดเลือดขาวชนิดนิวโทรฟิล

สารสังเคราะห์ไลป็อกซินเอโฟร์ได้ถูกนำมาศึกษาดูความเป็นพิษต่อนิวโทรฟิลโดยใช้ XTT assay สารนี้ในความเข้มข้น 0.1–100 ไมโครโมลาร์ ไม่พบว่ามีความเป็นพิษต่อเซลล์เม็ดเลือดขาวชนิดนิวโทรฟิลเมื่อ incubate สารนี้กับเซลล์เม็ดเลือดขาวชนิดนิวโทรฟิลนาน 4 ชั่วโมง อย่างไรก็ตาม พบว่าสารนี้ที่ความเข้มข้น 1000 ไมโครโมลาร์ ลดจำนวนเซลล์เม็ดเลือดขาวชนิดนิวโทรฟิลที่มีชีวิตลง 10 เปอร์เซนต์ สารนี้ในความเข้มข้น 10–100 ไมโครโมลาร์ สามารถยับยั้งการสร้างซุปเปอร์ออกไซด์แอนไอออนในเซลล์เม็ดเลือดขาวชนิดนิวโทรฟิลซึ่งเหนี่ยวนำด้วย fMLP สารนี้ไม่มีฤทธิ์ยับยั้งการสร้างเอนไซม์ไมอีโลเพอรอกซีเดสและไม่มีฤทธิ์ยับยั้งการหลั่งเอนไซม์อิลาสเทสในเซลล์เม็ดเลือดขาวชนิดนิวโทรฟิลจากการเหนี่ยวนำด้วยสาร fMLP

ฤทธิ์ในการยับยั้งการสร้างซุปเปอร์ออกไซด์แอนไอออนของนิวโทรฟิลซึ่งเหนี่ยวนำด้วย fMLP อาจเป็นกลไกหนึ่งที่เกี่ยวข้องในฤทธิ์ต้านการอักเสบของสารสังเคราะห์ไลป็อกซินเอโฟร์

คำสำคัญ: ไลป็อกซินเอโฟร์ ซุปเปอร์ออกไซด์แอนไอออน ไมอีโลเพอรอกซีเดส อิลาสเทส

Introduction

Lipoxin A4 (LXA4) is a metabolite of the arachidonic pathway. Epithelial-monocyte 15-Lipoxygenase (15-LO) produces 15(S)hydroperoxyeicosatetraneoic acid (15S-HpETE) from arachidonic acid, which can then be converted by neutrophil 5-Lipoxygenase (5-LO) to generate LXA4 (Figure 1). Production of lipoxin A4 by this pathway diverts metabolism of arachidonic acid from biosynthesis of pro-inflammatory leukotrienes. LXA4 can also be produced from leukotriene A4 (LTA4) by platelet 12-Lipoxygenase (12-LO). Other potential sources of lipoxins synthesis are linked to storage of precursors in membranes of inflammatory cells that may be released at the site of injury (1,2).

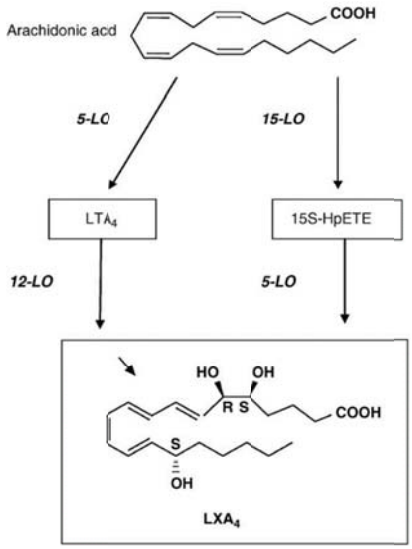

Figure 1. Biosynthesis of LXA4 (adapted from reference 1)

LXA4 possess its biological effect via G-protein coupled receptor (GPCR) named ALX/FPR2 receptor (3). A specific lipoxin (LX) recognition site was established in human polymorphonuclear neutrophils (PMN), myeloid, epithelial and mesenchymal cells (4).

Neutrophils are the "first responders" of the leukocytes. They begin to increase in number within seconds of an inflammatory response and then destroy invading microorganisms by phagocytosis, killing and digesting. The toxic oxygen metabolite, superoxide anion ($O_2^{\bullet-}$) are generated, however, if neutrophils are inappropriately activated, the excessive release of toxic oxygen metabolites can cause damage to host's own tissues (5). Myeloperoxidase (MPO), present in high concentrations in azurophillic granules of neutrophils, firstly released into the phagosome during granule-phagosome fusion and generates hypochlorous acid (HOCl) from H_2O_2 and Cl^- to defeat microorganisms. However, the excessive release of MPO can also damage host tissue and contributes to inflammatory tissue injury (6). Apart from $O_2^{\bullet-}$ and MPO, neutrophils elastase is released from neutrophils that migrate to the sites of infection or other damaged tissues during the early stages of inflammation, and is consequently a marker of inflammation (7).

LXA4 has been shown to act as anti-inflammatory agents in numerous models of disease and also shown to act as anti-inflammatory agent by inhibiting neutrophil function such as adhesion molecule expression, neutrophil chemotaxis and transmigration (8). Therefore, this study was further investigated the anti-inflammatory mechanism of this agent on $O_2^{\bullet-}$ generation, MPO production and elastase release in human neutrophils.

Materials and Methods

Materials

LXA4 (Item No.90410) was purchased from cayman chemical company, USA. Indomethacin, percoll, dextran, cytochalasin B, N-tert-butoxy-carbonyl-L-alanine-p-nitrophenyl ester (Boc-ala-ONp), 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-5-[(phenylamino) carboxy]-2H-tetrazolium hydroxide (XTT), n-methylphenazonium methosulfate (PMS), cytochrome C, tetramethylbenzidine (TMB), cytochalasin B, N-formyl-methionyl-leucyl-phenylalanine were purchased from Sigma, USA.

Isolation of human polymorphonuclear neutrophils

All procedures followed were in accordance with the Human Ethics Committee of Thammasat University No. 1 (Faculty of Medicine, Thailand) and with the Declaration of Helsinki, The Belmont Report, CIOMS Guideline and the International Practice (ICH-GCP).

Healthy volunteers

Seven healthy volunteers have signed an informed consent agreeing which approved by the Human Ethics Committee of Thammasat University No. 1 (Faculty of Medicine, Thailand) before blood collection. Inclusion criteria were healthy male or female subjects; aged 20-35 years. Exclusion criteria were subjects with anemia and diabetes mellitus.

Procedure

Venous blood was taken from the forearm of healthy volunteers who had not taken non-steroidal anti-inflammatory drugs for at least 7 days. Heparinized blood (10 U heparin/ml) was mixed with an equal volume of 3 % dextran in 0.9% NaCl and left to stand for 45 minutes at room temperature. Neutrophils were isolated by a discontinuous percoll gradient method (9). Their viability assessed by trypan blue exclusion. Neutrophils were resuspended either in warm RPMI 1640 medium with L-glutamine (without phenol red), for measurement of neutrophil cytotoxicity, or in phosphate buffered saline (PBS) containing Ca^{2+} and Mg^{2+} and kept at room temperature for measurement of $O_2^{\bullet-}$ generation, MPO production, and elastase release.

Neutrophil Viability Assay

XTT assay was used to measure the neutrophil viability (10). The living cells, but not the dead cells, reduce XTT by enzyme dehydrogenase in the active mitochondria to measurable quantities of XTT formazan. Neutrophils ($3x10^6$ cells/mL) were resuspended in RPMI 1640 with 10% heat-inactivated FBS. Neutrophils were incubated with LXA4 or indomethacin (final concentration of 0.1-1000 μM) for 4 hours. A mixture of XTT and 1% of PMS was added and incubated for 3 hours at 37°C with 5% CO_2 in humidified incubator. The XTT formazan product was then measured at A_{450} nm with a reference of A_{650} nm. Each reaction mixture was done in triplicate and the values were averaged.

Determination of $O_2^{\bullet-}$ Generation

$O_2^{\bullet-}$ generation was determined by the reduction of ferricytochrome C to ferrocytochrome C in the presence of cytochalasin B (10). Neutrophils ($3x10^6$ cells/ml) were resuspended in PBS (with Ca^{2+} and Mg^{2+}) containing cytochrome C and cytochalasin B. Neutrophils were pre-incubated with LXA4 or indomethacin (final concentration of 1-100 μM) for 10 minutes at 37°C. fMLP (final concentration of 100 nM) was then added and followed by further incubated for 10 minutes at 37°C. Supernatant from each tube was

measured at A_{550} nm. Each reaction mixture was done in triplicate and the values were averaged.

Determination of MPO production

MPO production was determined based on MPO-catalysed oxidation of its substrate by H_2O_2 (11). Neutrophil ($5x10^6$ cells/ml) were re-suspended in PBS (with Ca^{2+} and Mg^{2+}) containing cytochalasin B. Neutrophils were pre-incubated with LXA4 or indomethacin (final concentration of 1-100 μM) for 10 minutes at 37°C. fMLP (final concentration of 100 nM) was then added and further incubated for 10 minutes at 37°C. Supernatant was incubated with the TMB reaction mixture for 7 minutes at 37°C. The absorbance was measured at A_{450} nm. Each reaction mixture was done in triplicate and the values were averaged.

Determination of elastase release

The elastase release was assayed by the releasing of p-nitrophenol upon hydrolysis of Boc-ala-ONp (12). Neutrophils ($3x10^6$ cells/ml) were resuspended in PBS (with Ca^{2+} and Mg^{2+}) containing cytochalasin B. Neutrophils were pre-incubated with LXA4 or indomethacin (final concentration of 0.1-100 μM) for 10 minutes at 37°C. fMLP (final concentration of 100 nM) was then added and further incubated for 10 minutes at 37°C. Supernatant was incubated with Boc-ala-ONp for 20 minutes at 37°C. The released p-nitrophenol was measured at A_{414} nm. Each reaction mixture was done in triplicate and the values were averaged.

Statistical analysis

The results were presented as the mean±standard error of mean (S.E.M) of the averaged data taken from seven separate subjects. The data were analyzed by Student's t-test that was performed to compare the effects of LXA4 and indomethacin. Statistical differences will be considered significant when p-value < 0.05.

Results

Neutrophil Viability

Incubation of human neutrophils with LXA4 or indomethacin (final concentration of 0.1-100 μM) for 4 h had no effect on neutrophil viability. However, approximately a 10% reduction in neutrophil viability was observed after 4 h treatment of LXA4 and indomethacin at the concentration of 1000 μM, therefore this concentration did not use in the further assay.

Neutrophil $O_2^{\bullet-}$ Generation

LXA4 exerted an inhibition of fMLP-induced $O_2^{\bullet-}$ generation at the concentration of 10-100 μM. Indomethacin exhibited strong inhibition of fMLP-induced $O_2^{\bullet-}$ generation. The inhibitory effects on fMLP-induced $O_2^{\bullet-}$ generation of LXA4 at the concentration of 10-100 μM was significantly lower than that of indomethacin which is a reference compound. Indomethacin exhibited dose dependent inhibition of fMLP-induced neutrophil $O_2^{\bullet-}$ generation with IC50 of 56.5±2.5 μM (Table 1).

Table 1. Inhibitory effect of LXA4 and indomethacin on fMLP-induced neutrophil $O_2^{\bullet-}$ generation

Concentration (μM)	% Inhibition of Neutrophil $O_2^{\bullet-}$ generation	
	LXA4	Indomethacin
0.1	0.0±0.0	0.0±0.0
1	0.0±0.0	2.5±1.2
10	0.6±0.4	26.4±1.3*
100	14.9±3.5	82.8±3.3*
IC_{50}	> 100 μM	56.5±2.5*

Values are expressed as means±S.E.M. of seven different donors. Indomethacin was used as a reference compound. *p≤0.05 indicates a significant difference from LXA4.

Neutrophil MPO production

LXA4 at the concentration of 0.1-100 μM did not affect fMLP-induced MPO production in human neutrophils. Indomethacin exhibited dose dependent inhibition of fMLP-induced MPO production in neutrophils with IC50 of 67.2±2.4 μM (Table 2).

Table 2. Inhibitory effect of LXA4 and indomethacin on fMLP-induced human neutrophil MPO production

Concentration (μM)	% Inhibition of MPO production	
	LXA4	Indomethacin
0.1	0.0±0.0	3.9±2.7
1	0.0±0.0	9.5±1.8*
10	0.0±0.0	13.5±4.8*
100	0.0±0.0	72.3±1.2*
IC_{50}	-	67.2±2.4*

Values are expressed as means±S.E.M. of seven different donors. Indomethacin was used as a reference compound. *p≤0.05 indicates a significant difference from LXA4.

Neutrophil elastase release

LXA4 at the concentration of 0.1-100 μM did not affect fMLP-induced elastase release in human neutrophils. Indomethacin exhibited dose dependent inhibition of fMLP-induced elastase release in human neutrophils with IC50 of 63.9±5.3 μM (Table 3).

Table 3. Inhibitory effect of LXA4 and indomethacin on fMLP-induced human neutrophil elastase release

Concentration (μM)	% Inhibition of elastase release	
	LXA4	Indomethacin
0.1	0.0±0.0	3.0±1.2
1	0.0±0.0	15.6±2.7*
10	0.0±0.0	35.3±1.8*
100	0.0±0.0	77.2±4.9*
IC_{50}	-	63.9±5.3*

Values are expressed as means±S.E.M. of seven different donors. Indomethacin was used as a reference compound. *p≤0.05 indicates a significant difference from LXA4.

Discussion

The viability of neutrophils did not affected by LXA4 and indomethacin at concentration of 0.1-100 μM, when neutrophils at a concentration of $3x10^6$ cells/ml were incubated with these compounds for 4 hours. Indomethacin was used as a standard agent in the study of neutrophil functions. It is a potent nonselective cyclooxygenase (COX) inhibitor and may also inhibit phospholipase A and C, and reduce neutrophil migration (13). The formation of reactive oxygen species in neutrophils can be induced by naturally occurring agonists such as fMLP that involve G protein signaling (14).

In the present study, the ability of LXA4 to inhibit fMLP-induced neutrophil $O_2^{\bullet-}$ generation was investigated and the results demonstrated that LXA4 (concentration of 10-100 μM) inhibited $O_2^{\bullet-}$ generation activated by fMLP (100 nM). The neutrophil viability result showed that at the concentration of 10-100 μM, both LXA4 and indomethacin had no effect on neutrophil viability, therefore the inhibition of neutrophil $O_2^{\bullet-}$ generation by LXA4 and indomethacin was not due to their cytotoxic effects.

Apart from generating $O_2^{\bullet-}$, activated neutrophils also release several enzymes in the inflammatory process, such as MPO and elastase. MPO is an enzyme present in neutrophils and at a much lower concentration in monocytes and macrophages. It is known that the level of MPO activity is directly proportional to the neutrophil concentration in inflamed tissue. The measurement of the enzyme activity has been a quantitative and sensitive marker of chemotaxis and neutrophil infiltration in the inflammatory process.

Since fMLP could induce MPO production, the inhibition of the enzyme production might be associated with the blockage of the formyl peptide receptor or to the inhibition of the biochemical events within the cells. Previous studies have demonstrated that certain anti-inflammatory drugs are able to inhibit MPO activity and this inhibition may account for their anti-inflammatory effects. For example, anti-inflammatory drug aminopyrine was shown to inhibit conversion of H_2O_2 to HOCl by trapping MPO as an inactive redox intermediate compound II (15). MPO catalyzed the production of highly bactericidal HOCl from H_2O_2 and chloride. The present study demonstrated that LXA4 at the concentration of 0.1-100 μM did not inhibit fMLP-induced MPO production, however indomethacin which is a reference compound inhibited fMLP-induced MPO production with IC_{50} of 67.18±2.46 μM.

The inhibitory effect of the LXA4 on human neutrophil elastase release also investigated. LXA4 at the concentration of 0.1-100 μM did not affect fMLP-induced elastase release in human neutrophils, however indomethacin which is a reference compound inhibited fMLP-induced neutrophil elastase release with IC_{50} of 63.91±5.33 μM.

Conclusion

LXA4 exerted an inhibition of fMLP-induced $O_2^{\bullet-}$ generation at the concentration of 10-100 μM. The inhibitory effect of fMLP-induced $O_2^{\bullet-}$ generation of LXA4 at the concentration of 10-100 μM was significantly lower than that of indomethacin which is a reference compound. However, LXA4 did not affect fMLP-induced MPO production and elastase release in human neutrophils. Therefore, the inhibitory effect of $O_2^{\bullet-}$ generation on neutrophil responsiveness might be attributed, in part, to its anti-inflammatory activity.

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