(ทุนพัฒนาศักยภาพในการทำงานวิจัยของอาจารย์รุ่นใหม่)

สัญญาเลขที่ MRG6080047

รายงานวิจัยฉบับสมบูรณ์

โครงการกลไกการออกฤทธิ์ของพลัมบาจินและบทบาทของเอนไซม์ NADPH: quinone oxidoreductase 1 (NQO1) ในมะเร็งเต้านมที่ดื้อยาต้านฮอร์โมน

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กุมภาพันธ์ 2562

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัยและ
จุฬาลงกรณ์มหาวิทยาลัย

Abstract (บทคัดย่อ)

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Project Title : กลไกการออกฤทธิ์ของพลัมบาจินและบทบาทของเอนไซม์ NADPH: quinone oxidoreductase 1 (NQO1) ในมะเร็งเต้านมที่ดื้อยาต้านฮอร์โมน
(ภาษาอังกฤษ): Mechanisms of plumbagin as an anti-cancer agent and role of its enzyme NADPH: quinine oxidoreductase 1 (NQO1) in endocrine resistant breast cancer

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บทคัดย่อ

พลัมบาจินเป็นสารสกัดในกลุ่ม naphthoquinone ที่ได้จากธรรมชาติ มีฤทธิ์ต้านมะเร็งหลายชนิด โดยเฉพาะมะเร็งเต้านม ในการศึกษาก่อนหน้านี้พบว่าพลัมบาจินมีความเป็นพิษต่อเซลล์มะเร็งเต้านมที่ดื้อต่อยาต้านฮอร์โมนในความเข้มข้นระดับไมโครโมลาร์ เนื่องจากพลัมบาจินเป็นสาร naphthoquinone ที่ได้จากธรรมชาติ ซึ่งสารกลุ่มนี้จะถูกเอนไซม์ Nicotinamide adenine dinucleotide phosphate (NADPH) quinone oxidoreductase 1 (NQO1) ทำปฏิกิริยา reduction โดยพบว่าเอนไซม์ NQO1 มีการแสดงออกสูงขึ้นในเซลล์มะเร็งที่มีความรุนแรงหลายชนิด ในการศึกษานี้จึงศึกษาบทบาทของ NQO1 ในการออกฤทธิ์ของพลัมบาจินโดยใช้เซลล์ที่มีการลดการแสดงออกของ NQO1 และใช้ NQO1 inhibitor จากผลการศึกษาพบว่า มีการแสดงออกของ NQO1 เพิ่มมากขึ้นในเซลล์มะเร็งเต้านมที่ดื้อยาต้านฮอร์โมนและพลัมบาจินไม่มีผลต่อการเปลี่ยนแปลงการแสดงออกของระดับ NQO1 แต่พลัมบาจินไปเพิ่มการทำงาน (activity) ของ NQO1 การศึกษานี้ยังพบว่าฤทธิ์ของพลัมบาจินในการยับยั้งการเจริญเติบโต การลุกลามและการแสดงออกของยีนที่เกี่ยวข้องกับการดื้อยาต้านฮอร์โมนจะลดลงในเซลล์ที่มีการ

แสดงออกของ NQO1 ลดลงหรือในเซลล์ที่ได้ NQO1 inhibitor นอกจากนี้ฤทธิ์ของพลัมบาจินในการทำให้เซลล์มะเร็งมีการตายแบบ apoptosis และ การสร้าง reactive oxygen species (ROS) ก็ลดลงเมื่อ NQO1 น้อยลงหรือถูกยับยั้งการทำงาน ดังนั้นจึงสรุปได้ว่า กลไกการออกฤทธิ์ของพลัมบาจินในเซลล์มะเร็งเต้านมที่ดื้อยาต้านฮอร์โมนต้องอาศัยการทำงานของ NQO1

Abstract

Plumbagin (PLB) is a natural naphthoquinone with significant anticancer effects against several types of tumor cells including breast cancer. PLB exhibited potent cytotoxic activity at a micromolar concentration against endocrine-resistant breast cancer cell lines. Since PLB is a natural naphthoquinone compound, it can be reduced by the cytosolic Nicotinamide adenine dinucleotide phosphate (NADPH) quinone oxidoreductase 1 (NQO1) enzyme. NQO1 expression is high in many aggressive types of cancer. The roles of NQO1 in anti-cancer activity of PLB were investigated by using NQO1 knockdown cells and NQO1 inhibitor. The results showed that NQO1 was upregulated in endocrine-resistant cells. PLB did not change the expression of NQO1 but it was able to increase NQO1 activity. The inhibitory effects of PLB on cell proliferation, cell invasion and expression of tamoxifen resistant gene were attenuated in both NQO1 knockdown cells and NQO1 inhibited cells. Moreover, the effects of PLB to induce apoptosis and generate reactive oxygen species (ROS) were also lower in both NQO1 knockdown cells and NQO1 inhibited cells. Therefore, NQO1 activity is crucial for the action of PLB in endocrine-resistant breast cancer cells.

Keywords : plumbagin, endocrine resistance, NQO1, breast cancer
(คำหลัก)

Executive Summary

ความสำคัญและที่มาของปัญหา

มะเร็งเต้านมเป็นมะเร็งที่เกิดมากที่สุดในเพศหญิงและเป็นสาเหตุการตายลำดับสองรองจากมะเร็งปอด มะเร็งเต้านมชนิดที่มีการแสดงออกของตัวรับเอสโตรเจนเป็นมะเร็งเต้านมที่พบมากที่สุดประมาณร้อยละ 70 ของจำนวนผู้ป่วยโรคมะเร็งเต้านมทั้งหมด เนื่องจากฮอร์โมนเอสโตรเจนเป็นปัจจัยที่สำคัญในการเจริญเติบโตของมะเร็งเต้านมชนิดนี้จึงใช้ยาในกลุ่มยาต้านฮอร์โมนเอสโตรเจนเป็นการรักษาหลักในผู้ป่วยหลังการผ่าตัดเพื่อป้องกันการกลับเป็นซ้ำและการลุกลามของโรค โดยมียาในกลุ่ม Selective Estrogen Receptor Modulator (SERM) คือ tamoxifen และ Aromatase Inhibitor เป็นยาหลัก แต่อย่างไรก็ตามพบว่าร้อยละ 40 ของผู้ป่วยในระยะลุกลามมีการดื้อยาต้านฮอร์โมนโดยมีการกลับเป็นซ้ำและเกิดการลุกลามไปอวัยวะอื่นเป็นสาเหตุการเสียชีวิตที่สำคัญ เมื่อดื้อยาแล้วแพทย์อาจเปลี่ยนการรักษาเป็นยาต้านฮอร์โมนอีกกลุ่ม หรือยาตัวใหม่ ได้แก่ PI3K inhibitor และ CDK4/6 inhibitor แต่อย่างไรดีก็พบว่ายังเกิดการดื้อยาอยู่ รวมถึงผลข้างเคียงต่างๆ การรักษาผู้ป่วยกลุ่มนี้จึงมีตัวเลือกไม่มากนัก จากการศึกษาก่อนหน้าพบว่าสารสกัดจากธรรมชาติในกลุ่ม naphthoquinone ชื่อพลัมบาจิน จากพืช Plumbago indica มีฤทธิ์ที่ดีในการยับยั้งการเจริญเติบโตและการลุกลามของเซลล์มะเร็งเต้านมที่ดื้อต่อยาต้านฮอร์โมนในความเข้มข้นระดับไมโครโมลาร์ และมีฤทธิ์ที่ดีกว่าในเซลล์มะเร็งเต้านมที่ไม่ดื้อยา จากการศึกษาเบื้องต้นพบว่า มะเร็งเต้านมที่ดื้อต่อยาต้านฮอร์โมนมีการแสดงออกของเอนไซม์ NADPH: quinine oxidoreductase 1 (NQO1) มากกว่าในเซลล์มะเร็งเต้านมที่ไม่ดื้อยา โดย NQO1 มีหน้าที่ในกระบวนการ reduction ของสารกลุ่ม quinone ทำให้ได้ metabolite ในรูป stable หรือ unstable form ซึ่งทำให้เกิด reactive oxygen species (ROS) และ DNA adducts ซึ่งช่วยเพิ่มฤทธิ์ในการทำลายเซลล์มะเร็ง โดยพบว่าในเซลล์มะเร็งหลายชนิดมีการแสดงออกที่มากขึ้นของ NQO1 เช่นมะเร็งปอด ลำไส้ใหญ่ และตับอ่อน และพลัมบาจินก็เป็นสารกลุ่ม naphthoquinone ซึ่งเป็น substrate ของเอนไซม์นี้ การศึกษานี้จึงสนใจบทบาทของเอนไซม์ NQO1 ในการออกฤทธิ์ของพลัมบาจินและบทบาทในเซลล์มะเร็งเต้านมที่ดื้อต่อยาต้านฮอร์โมน

วัตถุประสงค์

- เพื่อศึกษากลไกการออกฤทธิ์ของสารพลัมบาจินในการต้านเซลล์มะเร็งที่ดื้อยาต้านฮอร์โมน
- เพื่อศึกษาบทบาทของเอนไซม์ NADPH: quinine oxidoreductase 1 (NQO1) ในเซลล์มะเร็งที่ดื้อยาต้านฮอร์โมนและบทบาทในการออกฤทธิ์ของพลัมบาจิน

ระเบียบวิธีวิจัย

ทำการทดลองในเซลล์มะเร็งเต้านมที่มีการแสดงออกของตัวรับเอสโตรเจน MCF-7 และเซลล์มะเร็งที่ดื้อต่อยาต้านฮอร์โมน ได้แก่ MCF-7/LCC2, MCF-7/LCC9 และศึกษาบทบาทของ NQO1 ต่อกลไกการออกฤทธิ์ของพลัมบาจิน โดยใช้ NQO1 siRNA และ NQO1 inhibitor

1. ศึกษาระดับการแสดงออกและการทำงานของ NQO1 และผลของพลัมบาจินต่อ NQO1 ด้วย Real-time PCR และ NQO1 activity assay
2. ศึกษาลักษณะของเซลล์มะเร็งที่เปลี่ยนแปลงไปเมื่อมีการลดการแสดงออกของ NQO1 โดยการ knock down NQO1 ด้วย si-RNA NQO1 แล้วนำเซลล์ไปดูผลต่อ

Proliferation ด้วย MTT assay

Invasion ด้วย matrigel invasion assay

Tamoxifen-related gene expression ด้วย Real-time PCR

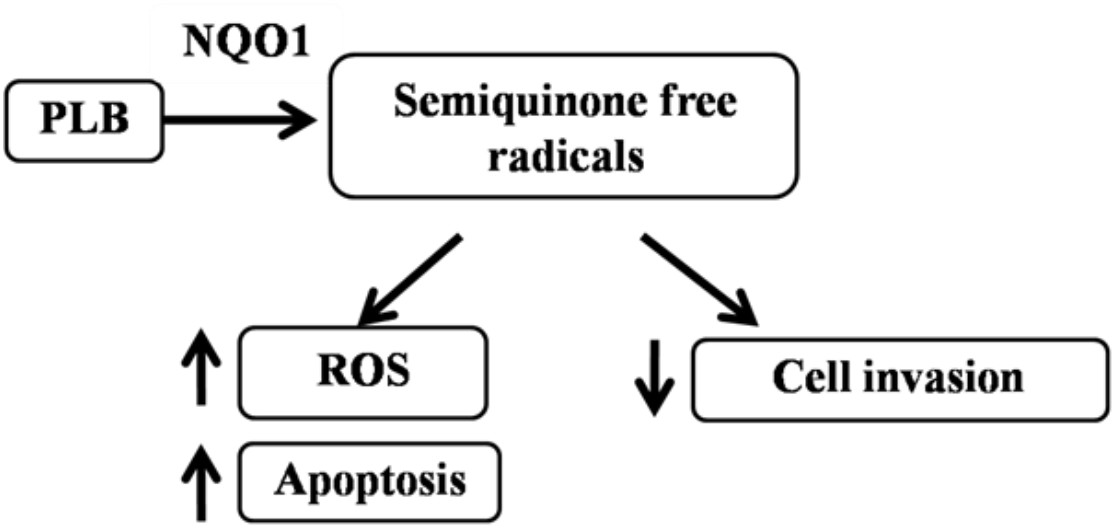
3. เพื่อศึกษาผลของ NQO1 ต่อการยับยั้งการเจริญเติบโตของเซลล์มะเร็งโดยพลัมบาจิน ด้วย MTT assay
4. เพื่อศึกษาผลของ NQO1 ต่อการสร้าง ROS โดยพลัมบาจิน ด้วย dichlorofluorescin diacetate (DCFDA) staining
5. เพื่อศึกษาผลของ NQO1 ต่อการเกิดเซลล์ apoptosis ของเซลล์มะเร็งโดยพลัมบาจิน ด้วย flow cytometry
6. เพื่อศึกษาผลของ NQO1 ต่อการยับยั้งการลุกลามของเซลล์มะเร็ง โดยพลัมบาจิน ด้วย matrigel invasion assay

ผลการทดลอง

เซลล์มะเร็งที่ดื้อยาต้านฮอร์โมนมีการแสดงออกของ NQO1 enzyme เพิ่มมากขึ้นจากเซลล์มะเร็งเต้านมที่ไม่ดื้อยา และเมื่อลดการทำงานของ NQO1 enzyme ลงด้วย NQO1 inhibitor และ knockdown NQO1 ด้วย SiRNA พบว่าผลการยับยั้งการเจริญเติบโต การสร้าง reactive oxygen species (ROS) และการลุกลามของสารพลัมบาจินต่อเซลล์มะเร็งเต้านมที่ดื้อต่อยาต้านฮอร์โมนลดลงอย่างมีนัยสำคัญทางสถิติ ซึ่ง NQO1 inhibitor ที่ใช้เป็นกลุ่มควบคุมไม่มีผลต่อการตายและการลุกลามของเซลล์ นอกจากนี้เมื่อลดการแสดงออกของ NQO1 ยังทำให้การเกิดเซลล์ตายโดยกระบวนการ apoptosis จากสารพลัมบาจินลดลงอย่างมีนัยสำคัญทางสถิติ ซึ่งสอดคล้องกับการแสดงออกของยีนที่เกี่ยวข้องกับการตายของเซลล์ แบบ apoptosis โดยพบว่าเมื่อมีการแสดงออกของ NQO1 ที่ลดลง ทำให้เมื่อให้พลัมบาจินในเซลล์มะเร็งที่ดื้อยาฮอร์โมนพบว่ามีการแสดงออกของ anti-apoptotic และ proapoptotic gene ที่เพิ่มขึ้น จากการศึกษานี้พบว่าการทดลองเป็นไปตามสมมติฐานคือ NQO1 enzyme มีผลต่อการออกฤทธิ์ของสารพลัมบาจินซึ่งเป็นสารที่เป็น substrate ของ enzyme นี้

สรุปและวิจารณ์ผลการทดลอง

การวิจัยก่อนหน้าพบว่า Nrf-2 มีผลต่อการแสดงออกของ NQO1 ใน squamous cell carcinoma แต่ในการศึกษานี้พบว่า Nrf-2 มีการแสดงออกลดลงเมื่อให้พลัมบาจินและเมื่อลดปริมาณการแสดงออก NQO1 หรือให้ NQO1 inhibitor การออกฤทธิ์ของพลัมบาจินเปลี่ยนแปลงไปในทางเดียวกันทั้งเซลล์มะเร็งที่ดื้อยาและไม่ดื้อยา และจากการศึกษานี้พบว่าการทำงานของพลัมบาจินต้องอาศัยการทำงานของ enzyme NQO1 ในเซลล์ที่ดื้อต่อยาต้านฮอร์โมน ซึ่งมีผลต่อทั้งการเจริญเติบโต การลุกลาม การสร้าง ROS และการตายของเซลล์แบบ apoptosis เป็นไปตามสมมติฐานงานวิจัย ดังนั้น NQO1 จึงมีบทบาทในการออกฤทธิ์ของพลัมบาจินในเซลล์มะเร็งที่ดื้อยาต้านฮอร์โมน ดังแสดงในรูป



ข้อเสนอแนะสำหรับงานวิจัยในอนาคต

ศึกษาการออกฤทธิ์ของพลัมบาจินในสัตว์ทดลองรวมไปถึงความปลอดภัย โดยเฉพาะเรื่องการแข็งตัวของเลือด เนื่องจากพลัมบาจินมีโครงสร้างคล้ายวิตามิน K

ผลงานวิจัย

Plumbagin had no effect on NQO1 expression but was able to increase NQO1 activity in endocrine-resistant breast cancer cells

NQO1 was up-regulated in various types of cancer such as colon, pancreas, breast and non-small cell lung cancers (Cresteil and Jaiswal, 1991; Siegel et al., 1998).The NQO1 mRNA level was significantly higher in the endocrine-resistant cell lines whereas NQO1 protein expression was significantly higher by 2.5 fold in only tamoxifen and fulvestrant-resistant MCF7/LCC9 cells **(figure 1A)**. PLB did not significantly change the NQO1 protein expression in any of the three cell lines **(figure 1B)**.

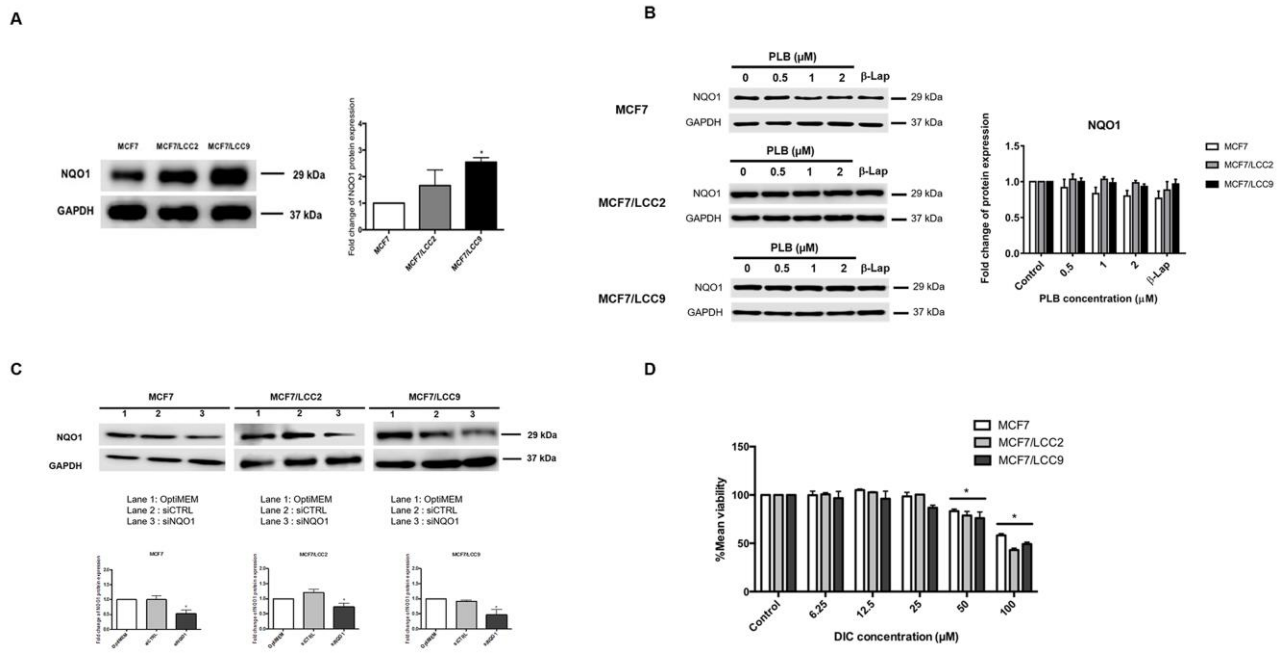


Figure 1. NQO1 expression and activity in the endocrine-resistant cell lines. A. Basal levels of NQO1 protein in wild-type MCF7, endocrine resistant breast cancer MCF7/LCC2 and MCF7/LCC9 cells are shown by Western blot. The data were normalized to GAPDH expression. The comparison of NQO1 expression of the 3 cell lines (mean ± SEM, n=3) are shown in the bar chart. * represents $p < 0.05$ vs. MCF7 cells. **B.** The levels of NQO1 after PLB treatment of the 3 cell lines (0.2% DMSO was used as the control and 1 μM β-lapachone (β-Lap) was used as the positive control, mean ± SEM, n=3) **C.** The level of NQO1 protein expression of the 3 cell lines after 24 hours of transfection with siNQO1 and negative control siRNA transfection using 1.5 μM of lipofectamine. The efficiency of the NQO1 knockdown by transient NQO1 siRNA transfection was confirmed by evaluating the levels of the protein expression. * represents $p < 0.05$ and ** represents $p < 0.01$ vs. siCTRL. **D.** Dicoumarol (DIC) is NQO1 inhibitor and is not toxic to breast cancer cells. The bar chart illustrates mean percentage and standard error of the mean of cell viability of MCF7 cells against DIC at increasing concentrations after 48 h incubation (mean ± SEM, n=3). The IC_{50} of DIC on MCF7, MCF7/LCC2, and MCF7/LCC9 is >100 μM, 88.50 μM ± 3.40, and 98.76 μM ± 3.03, respectively. * represents $p < 0.05$ vs. control (0.2% DMSO).

The activity of NQO1 was higher in endocrine resistant cells **(figure 2A)**. PLB significantly increased the NQO1 activity in the endocrine resistant MCF7/LCC2 and MCF7/LCC9 cells when compared to β-lapachone which was the other quinone compound used in the study **(figure 2C-D)**. The activity of NQO1 was lower in siNQO1

transfected endocrine-resistant cells when treated with PLB **(figure 2C-D)**. This effect was also observed in siNQO1 transfected wild-type MCF7 cells when the highest concentration of PLB (4 µM) was used **(figure 2B)**.

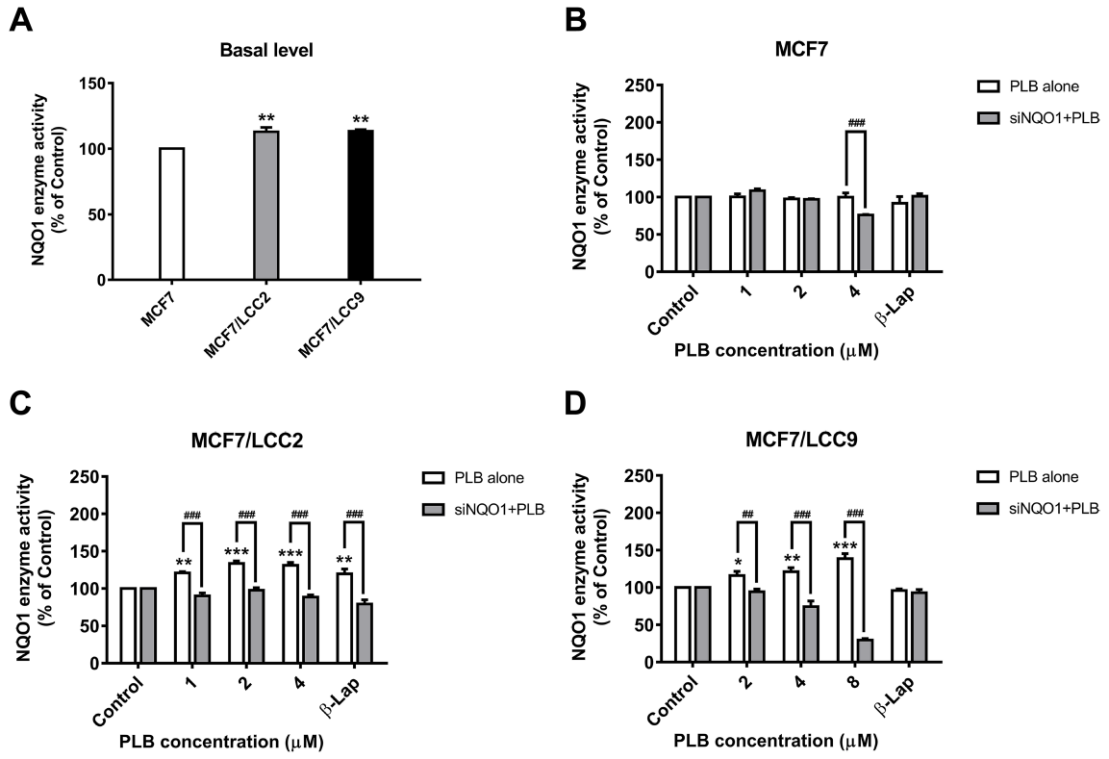


Figure 2. **The NQO1 enzymatic activity was increased by plumbagin in endocrine-resistant cells**. (A) The NQO1 activities of the 3 cell lines at baseline (mean ± SEM, n=3). ** represents $p < 0.01$ vs. MCF7 cells. The NQO1 activities of the 3 cell lines and cells transfected with siNQO1 after PLB treatment: MCF7 cells (B), MCF7/LCC2 (C) and MCF7/LCC9 (D) cells. * represents $p < 0.05$, ** represents $p < 0.01$, *** represents $p < 0.001$ vs. control (0.2% DMSO), and $^{\#\#}$ represents $p < 0.01$, $^{\#\#\#}$ represents $p < 0.001$ vs. PLB alone.

PLB has an inhibitory effect on Nrf-2 transcription factor that regulates NQO1 in squamous cell cancer (Pan et al., 2015). Because of this, the inhibitory effect of PLB on Nrf-2's expression level was assessed. The Nrf-2 mRNA expression was over-expressed in the endocrine-resistant cells **(figure 3A).** 4 μM and 8 μM of PLB significantly decreased the Nrf-2 expression of MCF7 cells. 1 μM, 2 μM and 4 μM of PLB also significantly decreased the Nrf-2 expression of MCF7/LCC2 cells. Likewise, 2 μM, 4 μM and 8 μM of PLB significantly decreased the Nrf-2 expression of MCF7/LCC9 cells **(figure 3B-D)**. However, the downregulation of NQO1 or inhibition of NQO1 activity attenuated the inhibitory effect of PLB on Nrf-2 expression in all cell lines **(figure 3B-D)**.

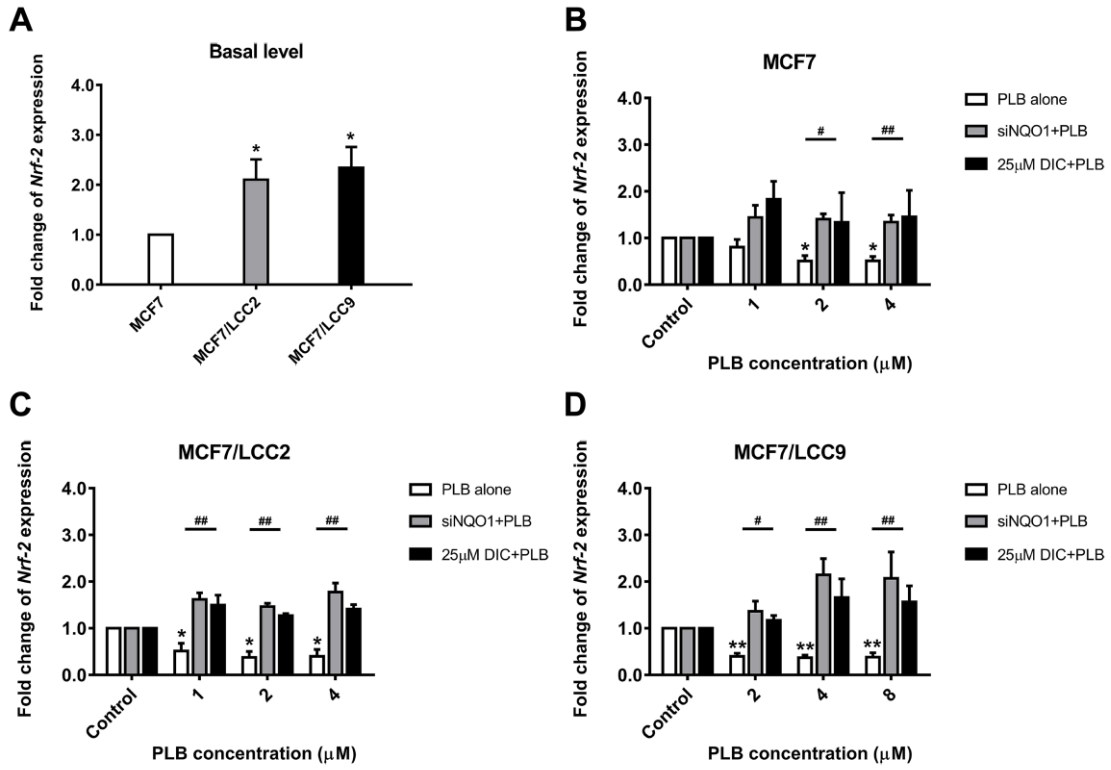


Figure 3. **The inhibitory effect of PLB on *Nrf-2* expression was lower when the NQO1 was downregulated or its activity was inhibited**. (A) The baseline Nrf-2 levels for the 3 cell lines by qRT-PCR analysis (mean ± SEM, n=3). * represents $p < 0.05$ vs MCF7 cells. The *Nrf-2* expression for PLB alone, 25 μM DIC pretreatment, and 24 h after all 3 cell lines were transfected with siNQO1: MCF7 (B) MCF7/LCC2 (C) and MCF7/LCC9 (D) cells.

* represents $p < 0.05$, ** represents $p < 0.01$ vs. control (0.2% DMSO), and $^{\#}$ represents $p < 0.05$, $^{\#\#}$ represents $p < 0.01$ vs. PLB alone.

Downregulation of NQO1 or inhibition of its activity resulted in attenuation of the inhibitory effects of plumbagin on cell proliferation

Micromolar concentrations of PLB was previously reported to inhibit the activity of the endocrine resistant cells (Sakunrangsit et al., 2016). The effect of NQO1 on PLB inhibitory effect was further investigated. The cytotoxic activity of PLB was lower in cells that were pretreated with NQO1 inhibitor or transfected with siNQO1. The IC_{50} of PLB in MCF-7 cells was 1.75 compared to 4.31 μM in MCF7 cells pretreated with NQO1 inhibitor and 2.03 μM in MCF7 cells transfected with siNQO1. The MCF7/LCC2 and MCF7/LCC9 cells showed the same pattern **(figure 4 and table 1)**. These results

showed that downregulation of NQO1 or inhibition of NQO1 activity attenuated the effects of PLB on cell proliferation in all cell lines.

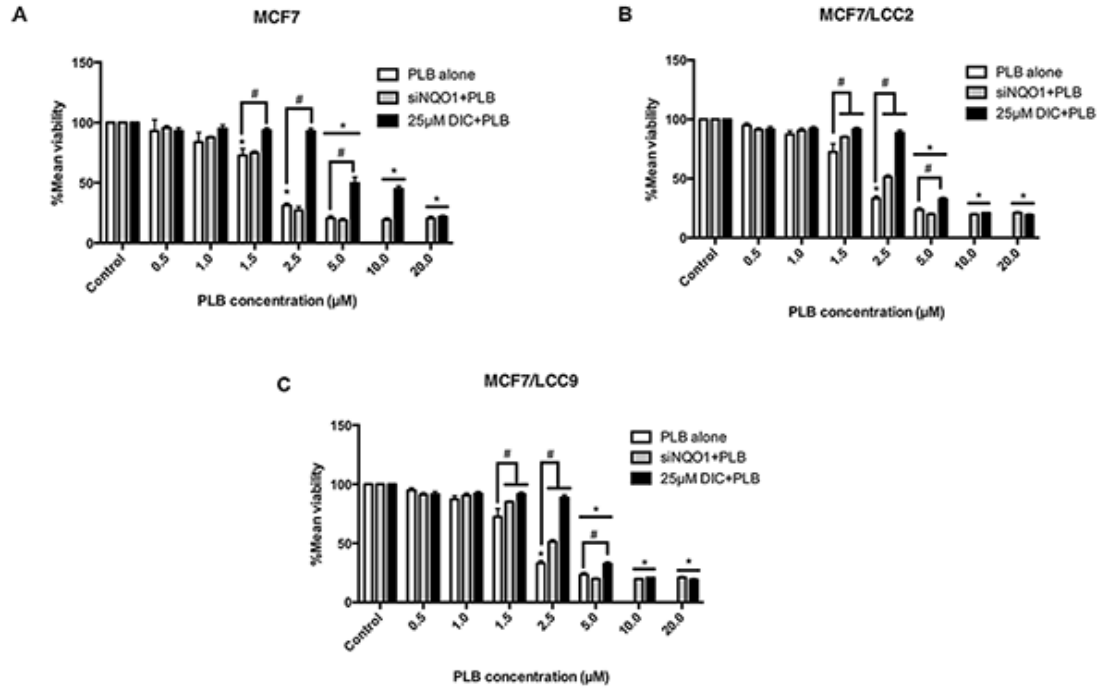


Figure 4. The inhibitory effect of PLB on cell proliferation was attenuated in endocrine-resistant cells when the cells were transfected with siNQO1 or pretreated with NQO1 inhibitor. The bar chart illustrates the cell viability (mean ± SEM, n=3) of the 3 cell lines after PLB treatment for MCF7 (A), MCF7/LCC2 (B) and MCF7/LCC9 (C) under the following 3 conditions: PLB alone, 25 μM DIC pretreatment, and siNQO1 transfection. * represents $p < 0.05$ vs. control (0.2% DMSO), # represents $p < 0.05$ vs. PLB alone.

Table 1. Comparison of IC_{50} of the 3 cell lines in 3 conditions; PLB alone, 25 μM DIC pretreatment, and siNQO1 transfection after PLB treatment (n = 3).

Conditions	IC_{50} (μM) MCF7	IC_{50} (μM) MCF7/LCC2	IC_{50} (μM) MCF7/LCC9
PLB alone	1.75	1.72	2.14
siNQO1 + PLB	2.03	2.55	4.15
Dicoumarol + PLB	4.31	4.30	4.11

NQO1 regulated the inhibitory effect of plumbagin on cell apoptosis and production of ROS in breast cancer cells

PLB has an apoptotic effect in prostate cancer cells, HER-2 over-expressed breast cancer cells and colon cancer cells (Chen et al., 2013; Kawiak et al., 2012; Powolny and Singh, 2008). However, the role of NQO1 in cancer cells remains unclear since it has been reported to assist apoptosis process and at the same time when used with different type of quinone compound, it has an opposite effect (Siegel et al., 2012). The effect of NQO1 on PLB induced-apoptosis was determined by annexin V-PE/7-ADD

staining. PLB significantly induced cell apoptosis when used at 2, 4 and 8 μM in MCF7, and at 4 and 8 μM in MCF7/LCC2 as well as MCF7/LCC9 cells. However, when the highest concentration of PLB (8 μM) was used, it was able to induce apoptosis in siNQO1 transfected cells and MCF7/LCC2 and MCF7/LCC9 cells that were pretreated with NQO1 inhibitor **(figure 5)**.

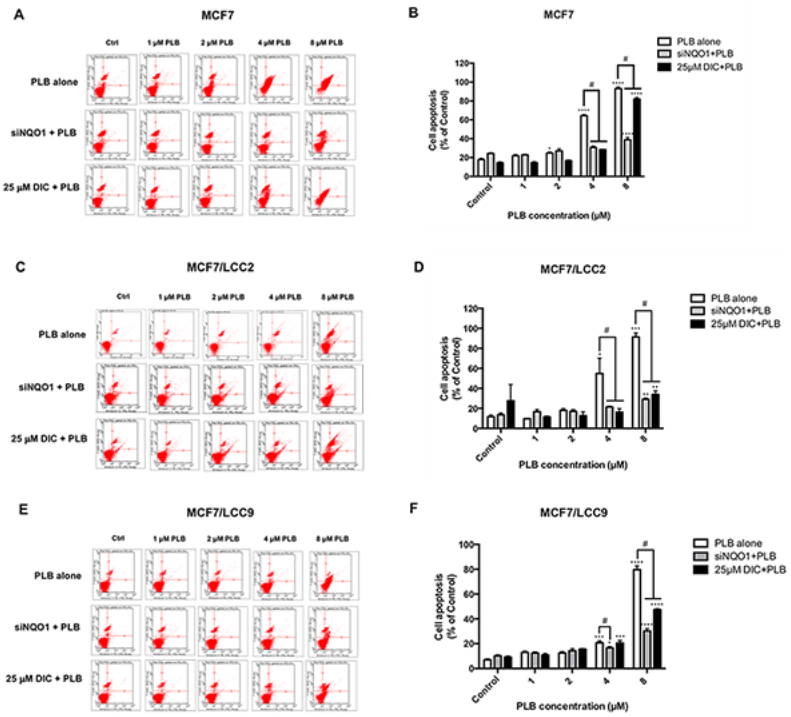


Figure 5. NQO1 was important for the inhibitory effect of PLB on cell apoptosis in endocrine-resistant cells when NQO1 expression was downregulated or its activity was inhibited. The flow cytogram and the percentage of cell apoptosis after MCF7 (A and B), MCF7/LCC2 (C and D), and MCF7/LCC9 (E and F) were treated with PLB (mean ± SEM, n=3) under the following 3 conditions: PLB alone, 25 μM DIC pretreatment, and siNQO1 transfection. * represents $p < 0.05$, *** represents $p < 0.001$

and **** represents $p < 0.0001$ vs. control (0.2% DMSO), and # represents $p < 0.05$ vs. PLB alone.

PLB decreased the mRNA expression of anti-apoptotic genes. The *BCL-2* mRNA expression was lower in MCF7/LCC2 cells treated with 2 and 4 μM of PLB and in MCF7/LCC9 cells treated with 2, 4 and 8 μM of PLB. The *BCL-xL* mRNA expression was lower in MCF7/LCC2 cells treated with 2 and 4 μM of PLB and in MCF7/LCC9 cells with 4 and 8 μM of PLB. However, the effect of PLB in MCF7 cells was not significant **(figure 6)**. The repressive effect of PLB was attenuated in the pretreated cells with NQO1 inhibitor and cells that were transfected with siNQO1. The levels of anti-apoptotic genes in the inhibitor-treated and knockdown NQO1 groups were also up-regulated when compared to the same PLB treatment **(figure 6)**.

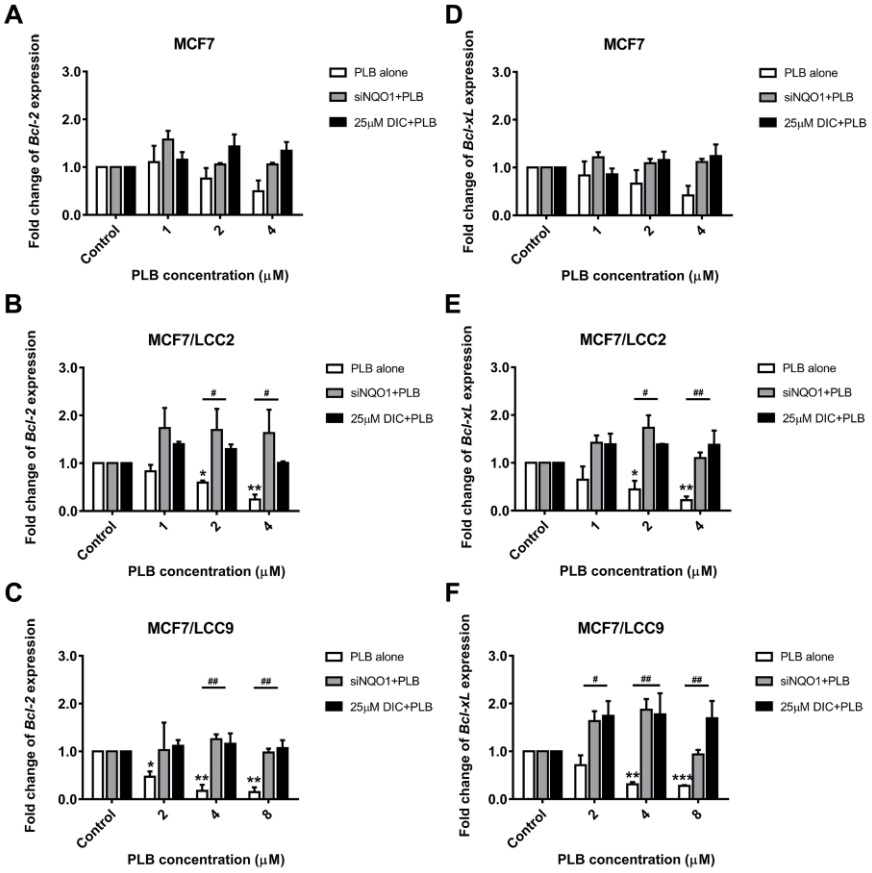


Figure 6. The inhibitory effects of PLB on apoptotic-related genes were lower in endocrine-resistant cells when NQO1 expression was downregulated or its activity was inhibited. The expression of *Bcl-2* gene by qRT-PCR for MCF7 (A), MCF7/LCC2 (B), MCF7/LCC9 cells (C) (mean ± SEM, n=3) under the following 3 conditions: PLB alone, 25 μM DIC pretreatment, and siNQO1 transfection. The *Bcl-xL* expression of MCF7 (D), MCF7/LCC2 (E), MCF7/LCC9 cells (F) under the following 3 conditions: PLB alone, 25 μM DIC pretreatment, and siNQO1 transfection. * represents $p < 0.05$, ** represents $p < 0.01$ and *** represents $p < 0.001$ vs. control (0.2% DMSO), and $^{\#}$ represents $p < 0.05$, $^{\#\#}$ represents $p < 0.01$ vs. PLB alone.

The study on cytotoxicity of PLB in ER-positive cell line (MCF-7 cells) showed that there was an inhibition of PI-5 kinase which is involved in the generation of ROS (Lee et al., 2012). NQO1 was shown to metabolize other types of quinone compounds as well as generate ROS and DNA adducts (Siegel et al., 2012). The association between the depletion of NQO1 and ROS formation has been reported to affect cell growth in A549 and H292 lung adenocarcinoma (Madajewski et al., 2016). Therefore, this property of NQO1 enzyme may be involved in the anti-cancer activity of PLB. The ROS production by PLB was assessed by DCFDA assay. 1, 4 and 8 μM of PLB was able to increase the ROS generation significantly in MCF7 and at all concentrations for MCF7/LCC2 and MCF7/LCC9 cells. However, the ability to generate ROS by PLB was lower in MCF7/LCC2 and MCF7/LCC9 cells that were pretreated with NQO1 inhibitor and cells that were transfected with siNQO1 **(figure 7)**.

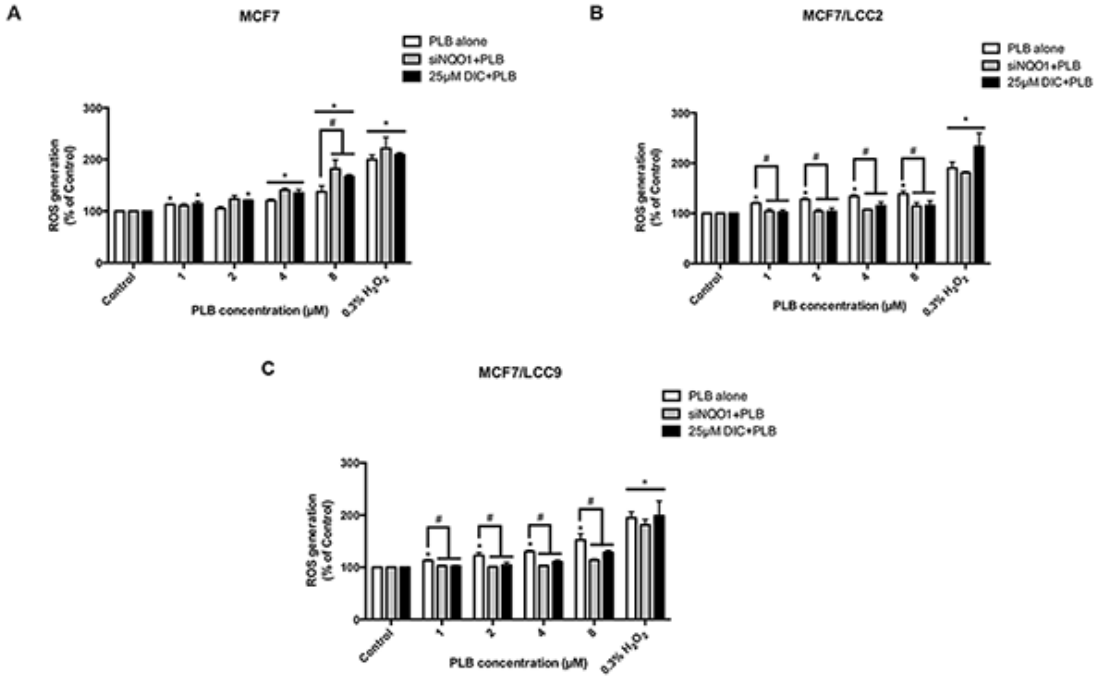


Figure 7. The ability of PLB to generate ROS was attenuated in endocrine-resistant cells when NQO1 expression was downregulated or its activity was inhibited. The comparison of PLB-induced reactive oxygen species (ROS) generation in MCF7 (A), MCF7/LCC2 (B) and MCF7/LCC9 (C) cells under the following 3 conditions: PLB alone, 25 μM DIC pretreatment, and siNQO1 transfection by DCFDA assay (means ± SEM, n = 3) * represents $p < 0.05$, ** represents $p < 0.01$ vs. control (0.2% DMSO), and # represents $p < 0.05$ vs. PLB alone.

NQO1 mediated the inhibitory effect of PLB on cell invasion in endocrine-resistant breast cancer cells

PLB has been reported to inhibit endocrine-resistant cell invasion (Sakunrangsit et al., 2016). In addition, depletion of NQO1 was reported to alter cell invasion in lung cancer cells (Madajewski et al., 2016). The effect of NQO1 on PLB was further studied in matrigel invasion assay. PLB significantly decreased cell invasion of MCF7/LCC2 cells and MCF7/LCC9 cells at every concentration studied. However, PLB was unable to significantly inhibit cell invasion in both MCF7/LCC2 cells and MCF7/LCC9 cells pretreated with NQO1 inhibitor and cells transfected with siNQO1 **(figure 8)**. Thus, the inhibitory effect of PLB on cell invasion was attenuated when the NQO1 was down-regulated or its activity was inhibited.

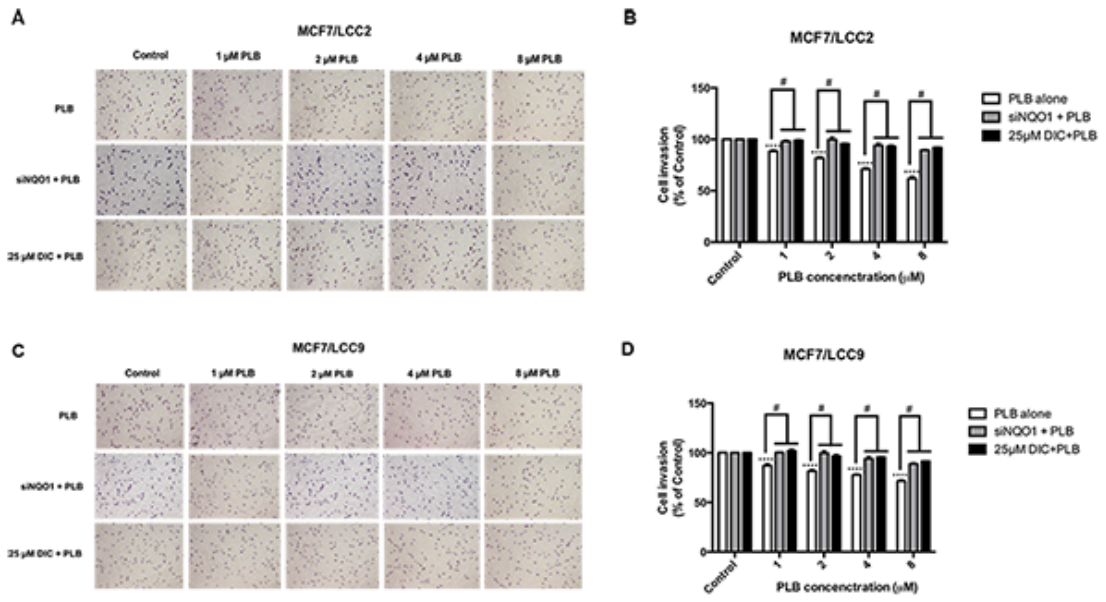


Figure 8. The inhibitory effect of PLB on cell invasion was abrogated in endocrine-resistant cells when NQO1 expression was downregulated or its activity was inhibited. The anti-invasive activity of PLB (percentage of the relative cell invasion) on MCF7/LCC2 (A and B) and MCF7/LCC9 (C and D) under the following 3 conditions: PLB alone, 25 μM DIC pretreatment, and siNQO1 transfection (mean ± SEM, n=3). **** represents $p < 0.0001$ vs. control (0.2% DMSO) and # represents $p < 0.05$ vs. PLB alone.

NQO1 played an important role in the inhibitory effects of plumbagin on tamoxifen resistant gene in breast cancer cells

Our previous study demonstrated the inhibitory effect of PLB on NCoA3, an ER-coactivator involved in tamoxifen-resistance (Sakunrangsit et al., 2016). We further investigated whether NQO1 was involved in the inhibitory effect of PLB on NCoA3 expression. 1 and 2 μM of PLB significantly decreased NCoA3 mRNA expression in both MCF7/LCC2 and MCF7/LCC9 cells. This repressive effect was attenuated and the level of NCoA3 mRNA was increased in both MCF7/LCC2 and MCF7/LCC9 cells pretreated with NQO1 inhibitor and cells transfected with siNQO1 **(figure 9)**.

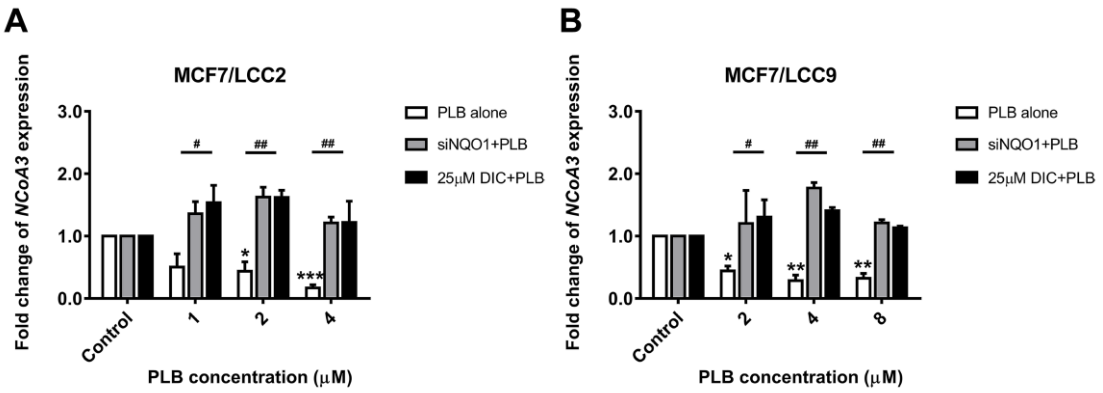


Figure 9. **The inhibitory effect of PLB on tamoxifen-resistant gene was attenuated in endocrine-resistant cells when the cells were pretreated with NQO1 inhibitor or transfected with siNQO1.** The *NCoA3* expression of the 2 endocrine-resistant cell lines by qRT-PCR (mean ± SEM, n=3) for MCF7/LCC2 (A) and MCF7/LCC9 cells (B) under the following 3 conditions: PLB alone, 25 μM DIC pretreatment, and siNQO1 transfection. * represents $p < 0.05$, ** represents $p < 0.01$ vs. control (0.2% DMSO), and $^{\#}$ represents $p < 0.05$, $^{\#\#}$ represents $p < 0.01$ vs. PLB alone.

Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ (ระบุชื่อผู้แต่ง ชื่อเรื่อง ชื่อวารสาร ปี เล่มที่ เลขที่ และหน้า) หรือผลงานตามที่คาดไว้ในสัญญาโครงการ

Pradubyat N., Sakunrangsit N., Mutirangura A., Ketchart W. (2019) NADPH: Quinone Oxidoreductase 1 (NQO1) Mediated Anti-Cancer Effects of Plumbagin in Endocrine Resistant Breast Cancer. Phytomedicine. [Manuscript submitted]

2. การนำผลงานวิจัยไปใช้ประโยชน์
   - เชิงวิชาการ (มีการพัฒนาการเรียนการสอน/สร้างนักวิจัยใหม่)

ได้นำผลงานวิจัยส่งเพื่อพิจารณาตีพิมพ์ในระดับนานานาชาติ และ นำงานวิจัยไปพัฒนาต่อยอดการวิจัยสารสกัดจากพืชที่มีฤทธิ์ต้านมะเร็งต่อไป รวมทั้งเป็นองค์ความรู้เพื่อใช้สอนนิสิตในระดับบัณฑิตศึกษา

3. อื่นๆ (เช่น ผลงานตีพิมพ์ในวารสารวิชาการในประเทศ การเสนอผลงานในที่ประชุมวิชาการ หนังสือ การจดสิทธิบัตร)

ได้นำผลงานวิจัยไปนำเสนอในการประชุมวิชาการระดับนานาชาติ ได้แก่งานประชุม 5th Annual Metastatic breast cancer conference Baltimore 2018 ณ John Hopkins Medicine ที่เมือง Baltimore ประเทศ สหรัฐอเมริการะหว่างวันที่ 15- 16 พฤศจิกายน พ.ศ. 2561

ภาคผนวก

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Section/Category: Cancer

Keywords: plumbagin; endocrine resistance; NQO1; breast cancer

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Abstract: Background: PLB is a natural naphthoquinone compound isolated from roots of Plumbago indica plant. Our previous study reported the inhibitory effect of Plumbagin (PLB) on human endocrine resistant breast cancer cell growth and cell invasion.
Hypothesis/Purpose: Since PLB is a naphthoquinone compound, it can be reduced by the cytosoloic NADPH: quinone oxidoreductase 1 (NQO1) enzyme. NQO1 expression is upregulated in various types of aggressive cancer including breast cancer. This study investigated the impact of NQO1 on anti-cancer effects of PLB in endocrine-resistant breast cancer cells.
Study Design: In vitro study
Methods: The roles of NQO1 in anti-cancer activity of PLB were investigated by using NQO1 knockdown cells and NQO1 inhibitor. To study the impact of NQO1 on inhibitory effects of PLB on cell viability, apoptosis, invasion and generation of ROS, MTT assays, annexin V-PE/7-ADD staining flow cytometry, matrigel invasion assays and DCFDA assays were performed in endocrine-resistant breast cancer cells. To study the mechanism of how NQO1 mediated PLB effects in tamoxifen response and apoptosis, levels of mRNA expression were evaluated by qRT-PCR.
Results: NQO1 was upregulated in endocrine-resistant cells. PLB did not change expression of NQO1 but it was able to increase NQO1 activity. The inhibitory effects of PLB on cell proliferation, cell invasion and expression of tamoxifen resistant gene were attenuated in NQO1 knockdown cells or when adding NQO1 inhibitor. Moreover, the effects of PLB to induce apoptosis and generate ROS were also decreased when inhibited NQO1 activity or decreased NQO1 expression.
Conclusion: NQO1 activity is critical for the action of PLB in endocrine-resistant breast cancer cells.

NADPH: Quinone Oxidoreductase 1 (NQO1) Mediated Anti-Cancer Effects of Plumbagin in Endocrine Resistant Breast Cancer

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Abstract

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Hypothesis/Purpose: Since PLB is a naphthoquinone compound, it can be reduced by the cytosoloic NADPH: quinone oxidoreductase 1 (NQO1) enzyme. NQO1 expression is upregulated in various types of aggressive cancer including breast cancer. This study investigated the impact of NQO1 on anti-cancer effects of PLB in endocrine-resistant breast cancer cells.

Study Design: This study is *in vitro* study using endocrine-resistant cell lines (MCF7/LCC2 and MCF7/LCC9 cells).

Methods: The roles of NQO1 in anti-cancer activity of PLB were investigated by using NQO1 knockdown cells and NQO1 inhibitor. To study the impact of NQO1 on inhibitory effects of PLB on cell viability, apoptosis, invasion and generation of ROS, MTT assays, annexin V-PE/7-ADD staining flow cytometry, matrigel invasion assays and DCFDA assays were performed in endocrine-resistant breast cancer cells. To study the mechanism of how NQO1 mediated PLB effects in tamoxifen response and apoptosis, levels of mRNA expression were evaluated by qRT-PCR.

Results: NQO1 was upregulated in endocrine-resistant cells. PLB did not change expression of NQO1 but it was able to increase NQO1 activity. The inhibitory effects of PLB on cell proliferation, cell invasion and expression of tamoxifen resistant gene were attenuated in NQO1 knockdown cells or when adding NQO1 inhibitor. Moreover, the effects of PLB to induce apoptosis and generate ROS were also decreased when inhibited NQO1 activity or decreased NQO1 expression.

Conclusion: NQO1 activity is critical for the action of PLB in endocrine-resistant breast cancer cells.

Keywords: plumbagin, endocrine resistance, NQO1, breast cancer

Abbreviations: DCFDA**;** 2′,7′-Dichlorofluorescin diacetate, DIC; dicoumarol, DMSO; dimethyl sulfoxide, FBS; fetal bovine serum, MEM; minimum essential medium, PLB; plumbagin, ROS; reactive oxygen species, 7-AAD; 7-Aminoactinomycin D

Introduction

Plumbagin (PLB) is isolated from the roots of *Plumbago indica*, a medicinal plant of the Plumbaginaceae family. PLB is a naphthoquinone compound that has demonstrated the ability to inhibit several tumor cells from growing including breast cancer. Quinones are widely found from natural products and serve as scaffold for cancer drugs. Quinone compounds are reduced by Nicotinamide adenine dinucleotide phosphate (NADPH); quinone oxidoreductase 1 (NQO1) enzyme that uses nicotinamide adenine dinucleotide phosphate (NADH) and its reduced form, NADPH as its cofactor (Glorieux et al., 2016). The NQO1 enzyme reduces quinone compounds to either a stable form or unstable form. A stable form is conjugated with uridine diphosphate (UDP)- glucoronic acid and excreted out of the cells. An unstable form generates reactive oxygen species (ROS). Therefore, NQO1 plays two opposite roles of detoxifying the metabolites or quinone intermediates while on the other hand, it can also generate ROS and DNA adducts (Siegel et al., 2012). NQO1 is reported to be upregulated in various types of cancer such as lung, colon, pancreas and breast cancers (Cresteil and Jaiswal, 1991; Siegel et al., 1998). Thus, the overexpression of NQO1 may assist the efficacy of bioactive quinone compounds. NQO1 is also reported to be involved with cell invasion in lung cancer (Madajewski et al., 2016). Moreover, clinical studies demonstrated a good correlation between NQO1 expression in tumor and the sensitivity to quinone-based chemotherapy (Fleming et al., 2002; Ough et al., 2005). Since PLB is a member of the quinone group, NQO1 enzyme may play a crucial role in inhibiting cell proliferation and invasion in endocrine-resistant cells. This study aimed to investigate the roles of NQO1 in the anti-cancer activity of PLB in endocrine-resistant breast cancer cells.

Materials and methods

Reagents and Antibodies

Plumbagin (PLB), Dicoumarol (DIC) and 3-4,5-Dimethyl-2-thiazolyl-2,5-diphenyl-2H-tetrazoliumbromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). PLB and DIC were dissolved in dimethyl sulfoxide (DMSO) (Sigma, USA). The concentration of PLB and DIC was 15 mM and 20 mM, respectively and stored at -20^{o}C.

Maintaining endocrine resistant cell lines

The MCF 7 cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). MCF7/LCC2 cell line is tamoxifen-resistant, while MCF7/LCC9 cell line is resistant to both tamoxifen and fulvestrant. Both of these cell lines are well characterized endocrine resistant cell lines and were obtained from Dr. Robert Clarke from the Lombardi Cancer Center, Georgetown University (Washington DC, USA). Cells were routinely cultured in Minimum Essential Media (MEM) containing 5% fetal bovine serum (FBS) (Gibco, USA) and maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2 incubator. Endocrine resistant cell lines were routinely checked to ensure that their resistance to tamoxifen did not wane with time by using MTT assay.

NQO1 siRNA transfection and NQO1 inhibitor treatment

NQO1 siRNA transfection

For transient NQO1 knockdown, siRNA-NQO1 (siNQO1) or scramble control siRNA was transiently transfected into MCF7/LCC2 or MCF7/LCC9 cells using Lipofectamine. Cells were harvested after 24 hours and analyzed for NQO1 expression. The expression of tamoxifen-resistant related genes was determined in siNQO1 transfected cells and was

compared to wild-type cells by western blot. Cells were counted by using cytotoxicity assay, invasion assay and RT-PCR for tamoxifen resistant genes. The methods of each experiment are described in detail in the following sections.

NQO1 inhibitor

A nontoxic concentration of dicoumarol (DIC), a NQO1 inhibitor, was assessed by MTT assay. Each cell line was treated with 25 µM of DIC for 24 hours. Cells were counted by using cytotoxicity assay, invasion assay and RT-PCR for tamoxifen resistant genes.

Real-time PCR

siNQO1 transfected cells treated with PLB and cells pretreated with NQO1 inhibitor were collected for RNA isolation. Total RNA was extracted by TRIzol reagent. One µg of total RNAs was converted to cDNA and amplified with specific primers to *NQO1* and *Nrf-2*. Real-time PCR was performed using SYBR Green supermix by StepOne™ Real-Time PCR System. GAPDH was used as an internal control.

Western blot

Cell lysates were used to perform western blots as previously described (Sakunrangsit et al., 2016). Membranes were probed with NQO1 primary antibody (Cell Signaling Technology, USA) overnight at 4°C and incubated with anti- mouse HRP-linked antibody in blocking solution. Band intensities of the protein were detected and the blots were analyzed as previously described (Sakunrangsit et al., 2016). Protein levels were normalized to the matching densitometry values of the GAPDH (Cell Signaling Technology, USA) which served as a loading control.

NQO1 activity assay

MCF7, MCF7/LCC2 or MCF7/LCC9 cells and siNQO1 transfected cells were treated with PLB for 6 h and then assayed to determine the activity of NQO1 enzyme. Cell pellets were collected from each cell line. Each pellet had 2×10^5 cells. Pellets were solubilized, extracted and measured for total protein by using NanoDrop One (Thermo Scientific, USA). NQO1 activity was evaluated by NQO1 activity assay kit (Abcam, Cambridge, MA, USA) (Madajewski et al., 2016). Absorbance of each sample was immediately measured at 440 nm by a microplate reader.

MTT assay

MCF7, MCF7/LCC2, and MCF7/LCC9 cells were seeded at a density of 5×10^3 cells per well into 96-well plates and maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2 incubator for overnight. Cells were washed and treated with various concentrations of PLB with and without NQO1 inhibitor for 24 and 48 h. siNQO1 transfected cells were also seeded and treated with PLB. MTT solution (5 mg/mL) in phosphate buffered saline (PBS) was added to each well and maintained for 4 h at 37°C in an incubator. The MTT assays were performed and analyzed as previously described (Sakunrangsit et al., 2016).

DCFDA assay

MCF7, MCF7/LCC2, and MCF7/LCC9 cells were labeled with 2′,7′-Dichlorofluorescin diacetate (DCFDA) (20 µM) and then incubated for an additional 24 hours with PLB and PLB in combination with NQO1 inhibitor according to the protocol. siNQO1 transfected cells were also treated with PLB. Cells were analyzed on a fluorescent plate reader. PLB oxidizes DCFDA to fluorescent DCF which mimics ROS activity.

Apoptotic assay

MCF7, MCF7/LCC2, and MCF7/LCC9 cells treated with PLB alone and PLB in combination with NQO1 inhibitor were collected and stained with fluorescein isothiocyanate-labeled annexin V-PE and 7-Aminoactinomycin D (7-AAD). siNQO1 transfected cells were also treated with PLB and stained with annexin V-PE/7-ADD. Fluorescence flow cytometry was used to analyze viable, apoptotic and dead cells. The expression levels of the anti-apoptotic genes such as *BCL-2* and *BCL-xL* were determined by RT-PCR.

Invasion assay

Cell invasion assay was performed using 8 μm pore size of transwell invasion inserts and 24-well plates (Corning, USA). MCF7/LCC2 and MCF7/LCC9 cells were treated with a fixed concentration of PLB or 0.1% DMSO (vehicle control) with or without NQO1 inhibitor. siNQO1 transfected cells were treated with PLB. The invasion assays were performed as previously described (Sakunrangsit et al., 2016).

Statistical analysis

Data from at least three independent experiments of which each experiment was done in triplicates were represented as mean ± SEM. Student's t-test was used to detect any significant differences between the two groups. Comparisons of multiple groups were determined by one-way ANOVA followed by the Tukey's test. The statistical analysis was calculated by SPSS 22.0 software (Chicago, IL, USA). *P*- value < 0.05 was considered to be statistically significant.

Results

Plumbagin had no effect on NQO1 expression but was able to increase NQO1 activity in endocrine-resistant breast cancer cells

The NQO1 mRNA level was significantly higher in the endocrine-resistant cell lines whereas NQO1 protein expression was significantly higher by 2.5 fold in only tamoxifen and fulvestrant-resistant MCF7/LCC9 cells **(figure 1A)**. PLB did not significantly change the NQO1 protein expression in any of the three cell lines **(figure 1B)**.

The activity of NQO1 was higher in endocrine resistant cells **(figure 2A)**. PLB significantly increased the NQO1 activity in the endocrine resistant MCF7/LCC2 and MCF7/LCC9 cells when compared to β-lapachone which was the other quinone compound used in the study **(figure 2C-D)**. The activity of NQO1 was lower in siNQO1 transfected endocrine-resistant cells when treated with PLB **(figure 2C-D)**. This effect was also observed in siNQO1 transfected wild-type MCF7 cells when the highest concentration of PLB (4 μM) was used **(figure 2B)**.

PLB has an inhibitory effect on Nrf-2 transcription factor that regulates NQO1 in squamous cell cancer (Pan et al., 2015). The Nrf-2 mRNA expression was over-expressed in the endocrine-resistant cells **(Supplementary figure 3A).** PLB significantly decreased the Nrf-2 expression of MCF7, MCF7/LCC2 and MCF7/LCC9 cells. However, the downregulation of NQO1 or inhibition of NQO1 activity attenuated the inhibitory effect of PLB on Nrf-2 expression in all cell lines **(Supplementary figure 3B-D)**.

Downregulation of NQO1 or inhibition of its activity resulted in attenuation of the inhibitory effects of plumbagin on cell proliferation

The cytotoxic activity of PLB was lower in cells that were pretreated with NQO1 inhibitor or transfected with siNQO1. The IC_{50} of PLB in MCF-7 cells was 1.75 compared to 4.31 μM in MCF7 cells pretreated with NQO1 inhibitor and 2.03 μM in MCF7 cells transfected with siNQO1. The MCF7/LCC2 and MCF7/LCC9 cells showed the same pattern **(figure 3 and table 1)**. These results showed that downregulation of NQO1 or inhibition of NQO1 activity attenuated the effects of PLB on cell proliferation in all cell lines.

NQO1 regulated the inhibitory effect of plumbagin on cell apoptosis and production of ROS in breast cancer cells

The effect of NQO1 on PLB induced-apoptosis was determined by annexin V-PE/7-ADD staining. PLB significantly induced cell apoptosis when used at 2, 4 and 8 μM in MCF7, and at 4 and 8 μM in MCF7/LCC2 as well as MCF7/LCC9 cells. However, when the highest concentration of PLB (8 μM) was used, it was able to induce apoptosis in siNQO1 transfected cells and MCF7/LCC2 and MCF7/LCC9 cells that were pretreated with NQO1 inhibitor **(figure 4)**.

PLB decreased the mRNA expression of anti-apoptotic genes. The *BCL-2* mRNA expression was lower in MCF7/LCC2 cells treated with 2 and 4 μM of PLB and in MCF7/LCC9 cells treated with 2, 4 and 8 μM of PLB. The *BCL-xL* mRNA expression was lower in MCF7/LCC2 cells treated with 2 and 4 μM of PLB and in MCF7/LCC9 cells with 4 and 8 μM of PLB. However, the effect of PLB in MCF7 cells was not significant **(figure 5)**. The repressive effect of PLB was attenuated in the pretreated cells with NQO1 inhibitor and cells that were transfected with siNQO1. The levels of anti-apoptotic genes in the inhibitor-treated and knockdown NQO1 groups were also up-regulated when compared to the same

PLB treatment **(figure 5)**. The study on cytotoxicity of PLB in ER-positive cell line (MCF-7 cells) showed that there was an inhibition of PI-5 kinase which is involved in the generation of ROS (Lee et al., 2012)**.** NQO1 was shown to metabolize other types of quinone compounds as well as generate ROS and DNA adducts (Siegel et al., 2012). The association between the depletion of NQO1 and ROS formation has been reported to affect cell growth in A549 and H292 lung adenocarcinoma (Madajewski et al., 2016)**.** Therefore, this property of NQO1 enzyme may be involved in the anti-cancer activity of PLB. The ROS production by PLB was assessed by DCFDA assay. 1, 4 and 8 μM of PLB was able to increase the ROS generation significantly in MCF7 and at all concentrations for MCF7/LCC2 and MCF7/LCC9 cells. However, the ability to generate ROS by PLB was lower in MCF7/LCC2 and MCF7/LCC9 cells that were pretreated with NQO1 inhibitor and cells that were transfected with siNQO1 **(figure 6)**.

NQO1 mediated the inhibitory effect of PLB on cell invasion in endocrine-resistant breast cancer cells

PLB has been reported to inhibit endocrine-resistant cell invasion (Sakunrangsit et al., 2016). In addition, depletion of NQO1 was reported to alter cell invasion in lung cancer cells (Madajewski et al., 2016). The effect of NQO1 on PLB was further studied in matrigel invasion assay. PLB significantly decreased cell invasion of MCF7/LCC2 cells and MCF7/LCC9 cells at every concentration studied. However, PLB was unable to significantly inhibit cell invasion in both MCF7/LCC2 cells and MCF7/LCC9 cells pretreated with NQO1 inhibitor and cells transfected with siNQO1 **(figure 7)**. Thus, the inhibitory effect of PLB on cell invasion was attenuated when the NQO1 was down-regulated or its activity was inhibited.

NQO1 played an important role in the inhibitory effects of plumbagin on tamoxifen resistant gene in breast cancer cells

Our previous study demonstrated the inhibitory effect of PLB on NCoA3, an ER-coactivator involved in tamoxifen-resistance (Sakunrangsit et al., 2016). We further investigated whether NQO1 was involved in the inhibitory effect of PLB on NCoA3 expression. 1 and 2 μM of PLB significantly decreased NCoA3 mRNA expression in both MCF7/LCC2 and MCF7/LCC9 cells. This repressive effect was attenuated and the level of NCoA3 mRNA was increased in both MCF7/LCC2 and MCF7/LCC9 cells pretreated with NQO1 inhibitor and cells transfected with siNQO1 **(figure 8)**.

Discussion

PLB significantly inhibits the growth of several tumor cells such as gastric, liver, lung, pancreatic, prostate, colon and breast cancers (Ahmad et al., 2008; Chen et al., 2013; Hafeez et al., 2015; Lai et al., 2012; Manu et al., 2011). PLB had no apoptotic-inducing effect in normal human cells and in mice (Ahmad et al., 2008; Aziz et al., 2008; Sumsakul et al., 2014). However, the efficacy of PLB has not yet been studied in human endocrine-resistant breast cancer models and its mechanism of action is still unclear. Sakunrangsit et al. investigated the cytotoxicity and reversal effects of PLB in human endocrine- resistant breast cancer cells (Sakunrangsit et al., 2016). The inhibitory effect of PLB in endocrine-resistant cells was in micromolar concentration range, which was almost the same level as wild-type ER positive breast cancer cell line. Moreover, our previous study also demonstrated that PLB reduced NCoA3 which is an ER co-activator that can increase the expression of tamoxifen in tamoxifen resistant cells (Sakunrangsit et al., 2016).

NQO1 is an inducible two-electron oxidoreductase enzyme which plays vital roles in phase II detoxification and bioactivation of various DNA-damaging quinone compounds (Ross et al., 2000). NQO1 can function as a protective factor or anti-oxidant enzyme against mutagenicity and carcinogenicity when it metabolizes certain quinone compounds or carcinogens into stable metabolites that are excreted out of the body. NQO1 can also metabolize other types of quinone compounds as well as generate ROS and DNA adducts (Siegel et al., 2012). These properties of NQO1 enzyme may be useful for anti-cancer drugs which are quinone-based compounds including PLB.

The expression level of NQO1 is different between normal and tumor tissues for both protein level and enzyme activity (Hungermann et al., 2011). Overexpression of NQO1 in oxidative stress-resistant breast cancer cells compared to the parental cell line has been reported (Glorieux et al., 2016). Madajewski et al. reported the association of the depletion of NQO1 with ROS formation which inhibited cell growth and decreased cell invasion in A549 and H292 lung adenocarcinoma (Madajewski et al., 2016). There are some clinical studies which demonstrated that the NQO1 expression in tumors was associated with sensitivity to quinone-based chemotherapy (Fleming et al., 2002; Ough et al., 2005). Moreover, in a recent study, β-lapachone, a quinone compound, could inhibit cell invasion in NQO1-positive breast cancer cells (Yang et al., 2017). In addition, PLB has been reported to inhibit Nrf-2 in squamous cell carcinoma (Pan et al., 2015). Nrf-2 is a transcription factor that regulates oxidative stress by activating anti-oxidant enzyme and was observed to be over-expressed in cancer cells (DeNicola et al., 2011). One of the anti-oxidant genes that is regulated by Nrf-2 is *NQO1*. However, there was no report on the impact of NQO1 in endocrine-resistant breast cancer.

The result of this study demonstrated that the over-expression of NQO1 was observed only in fulvestrant and tamoxifen-resistant breast cancer cell line (MCF7/LCC9). In addition,

NQO1 activity was higher in both endocrine-resistant cell lines. Even though, PLB did not alter NQO1 expression in breast cancer cells, however it increased NQO1 activity in endocrine-resistant cells. These results were consistent with other experiments that was conducted in endocrine-resistant cells pretreated with NQO1 inhibitor or transfected with siNQO1 to knockdown the NQO1 expression. The inhibitory effects of PLB on cell proliferation, cell invasion, apoptosis and the ability to generate ROS were significantly attenuated in endocrine-resistant cells. Since wild-type ER-positive cells still expressed lower level of NQO1, the attenuated ability was still observed in some experiments but the effects were not significant when compared with the resistant cells. Moreover, PLB was able to reduce NCoA3 expression in endocrine-resistant cells which is an important factor in tamoxifen resistance (Sakunrangsit et al., 2016). The inhibitory effect of PLB on NCoA3 expression was also attenuated when NQO1 was downregulated or its activity was inhibited. Therefore, the NQO1 activity may be involved in the mechanisms of endocrine resistance in breast cancer. This is the first study to demonstrate the important role of NQO1 in anti-hormonal resistance.

Although IC_{50} of PLB in MCF7/LCC2 cells was not significantly altered in cells that were transfected with siNQO1, however the inhibitory effects of PLB became weak. After the cells were treated with PLB, the activity of NQO1 was significantly higher even though its expression did not alter. This finding suggested that the NQO1 protein in this cell line was hyperactive. Therefore, this effect may have been due to the reduction of NQO1 activity rather than its expression. Moreover, PLB significantly decreased Nrf-2 expression in both wild-type and endocrine-resistant cells. Nrf-2 is the transcription factor that regulates NQO1 gene expression. However, this mechanism was not specific with endocrine-resistant cells since the same effect was also observed in wild-type MCF7 cells.

In summary, NQO1 is crucial for PLB's anti-cancer effects because PLB is the substrate of NQO1 enzyme that can be reduced to become an unstable form that generates ROS in endocrine-resistant cells. This in turn makes PLB a better anti-cancer agent against endocrine-resistant breast cancer cells. Thus, the results of this study provide a new insight for the treatment of not only tamoxifen-resistant breast cancer, but also other over-expressed NQO1 cancers. Further studies of PLB's molecular mechanism and animal models are still required to support the use of PLB as a treatment option for endocrine-resistant breast cancer patients.

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Conflict of interest

The authors declare that there is no conflict of interest.

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Table 1. Comparison of IC_{50} of the 3 cell lines in 3 conditions; PLB alone, 25 μM DIC pretreatment, and siNQO1 transfection after PLB treatment (n = 3).

Figure 1. NQO1 expression and activity in the endocrine-resistant cell lines. A. Basal levels of NQO1 protein in wild-type MCF7, endocrine resistant breast cancer MCF7/LCC2 and MCF7/LCC9 cells are shown by Western blot. The data were normalized to GAPDH expression. The comparison of NQO1 expression of the 3 cell lines (mean ± SEM, n=3) are shown in the bar chart. * represents $p < 0.05$ vs. MCF7 cells. **B.** The levels of NQO1 after PLB treatment of the 3 cell lines (0.2% DMSO was used as the control and 1 μM β-lapachone (β-Lap) was used as the positive control, mean ± SEM, n=3)

Figure 2. **The NQO1 enzymatic activity was increased by plumbagin in endocrine-resistant cells**. (A) The NQO1 activities of the 3 cell lines at baseline (mean ± SEM, n=3). ** represents $p < 0.01$ vs. MCF7 cells. The NQO1 activities of the 3 cell lines and cells transfected with siNQO1 after PLB treatment: MCF7 cells (B), MCF7/LCC2 (C) and MCF7/LCC9 (D) cells. * represents $p < 0.05$, ** represents $p < 0.01$, *** represents $p < 0.001$ vs. control (0.2% DMSO), and $^{\#\#}$ represents $p < 0.01$, $^{\#\#\#}$ represents $p < 0.001$ vs. PLB alone.

Figure 3. The inhibitory effect of PLB on cell proliferation was attenuated in endocrine-resistant cells when the cells were transfected with siNQO1 or pretreated with NQO1 inhibitor. The bar chart illustrates the cell viability (mean ± SEM, n=3) of the 3 cell lines after PLB treatment for MCF7 (A), MCF7/LCC2 (B) and MCF7/LCC9 (C) under the following 3 conditions: PLB alone, 25 μM DIC pretreatment, and siNQO1 transfection. * represents $p < 0.05$ vs. control (0.2% DMSO), # represents $p < 0.05$ vs. PLB alone.

Figure 4. NQO1 was important for the inhibitory effect of PLB on cell apoptosis in endocrine-resistant cells when NQO1 expression was downregulated or its activity was inhibited. The flow cytogram and the percentage of cell apoptosis after MCF7 (A and B), MCF7/LCC2 (C and D), and MCF7/LCC9 (E and F) were treated with PLB (mean ± SEM, n=3) under the following 3 conditions: PLB alone, 25 μM DIC pretreatment, and siNQO1 transfection. * represents $p < 0.05$, *** represents $p < 0.001$ and **** represents $p < 0.0001$ vs. control (0.2% DMSO), and # represents $p < 0.05$ vs. PLB alone.

Figure 5. The inhibitory effects of PLB on apoptotic-related genes were lower in endocrine-resistant cells when NQO1 expression was downregulated or its activity was inhibited. The expression of *Bcl-2* gene by qRT-PCR for MCF7 (A), MCF7/LCC2 (B), MCF7/LCC9 cells (C) (mean ± SEM, n=3) under the following 3 conditions: PLB alone, 25 μM DIC pretreatment, and siNQO1 transfection. The *Bcl-xL* expression of MCF7 (D), MCF7/LCC2 (E), MCF7/LCC9 cells (F) under the following 3 conditions: PLB alone, 25 μM DIC pretreatment, and siNQO1 transfection. * represents $p < 0.05$, ** represents $p < 0.01$ and *** represents $p < 0.001$ vs. control (0.2% DMSO), and $^{\#}$ represents $p < 0.05$, $^{\#\#}$ represents $p < 0.01$ vs. PLB alone.

Figure 6. The ability of PLB to generate ROS was attenuated in endocrine-resistant cells when NQO1 expression was downregulated or its activity was inhibited. The comparison of PLB-induced reactive oxygen species (ROS) generation in MCF7 (A), MCF7/LCC2 (B) and MCF7/LCC9 (C) cells under the following 3 conditions: PLB alone, 25 μM DIC pretreatment, and siNQO1 transfection by DCFDA assay (means ± SEM, n = 3) * represents $p < 0.05$, ** represents $p < 0.01$ vs. control (0.2% DMSO), and # represents $p < 0.05$ vs. PLB alone.

Figure 7. The inhibitory effect of PLB on cell invasion was abrogated in endocrine-resistant cells when NQO1 expression was downregulated or its activity was inhibited. The anti-invasive activity of PLB (percentage of the relative cell invasion) on MCF7/LCC2 (A and B) and MCF7/LCC9 (C and D) under the following 3 conditions: PLB alone, 25 μM DIC pretreatment, and siNQO1 transfection (mean ± SEM, n=3). **** represents $p < 0.0001$ vs. control (0.2% DMSO) and # represents $p < 0.05$ vs. PLB alone.

Figure 8. **The inhibitory effect of PLB on tamoxifen-resistant gene was attenuated in endocrine-resistant cells when the cells were pretreated with NQO1 inhibitor or transfected with siNQO1.** The *NCoA3* expression of the 2 endocrine-resistant cell lines by qRT-PCR (mean ± SEM, n=3) for MCF7/LCC2 (A) and MCF7/LCC9 cells (B) under the following 3 conditions: PLB alone, 25 μM DIC pretreatment, and siNQO1 transfection. * represents $p < 0.05$, ** represents $p < 0.01$ vs. control (0.2% DMSO), and $^{\#}$ represents $p < 0.05$, $^{\#\#}$ represents $p < 0.01$ vs. PLB alone.

Table1

Conditions	IC_{50} (µM) MCF7	IC_{50} (µM) MCF7/LCC2	IC_{50} (µM) MCF7/LCC9
PLB alone	1.75	1.72	2.14
siNQO1 + PLB	2.03	2.55	4.15
Dicoumarol + PLB	4.31	4.30	4.11

Figure1

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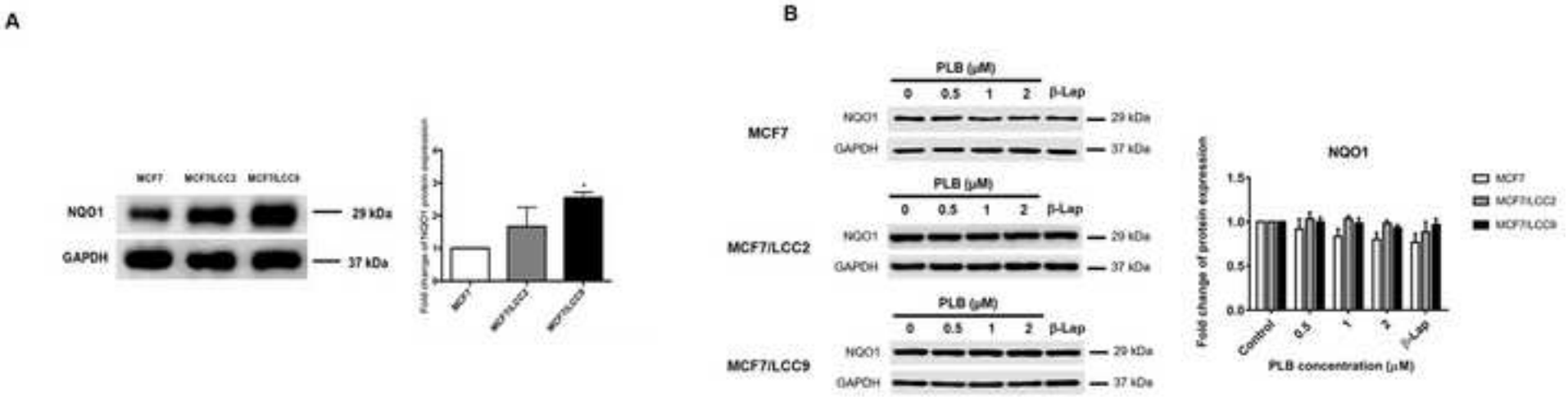

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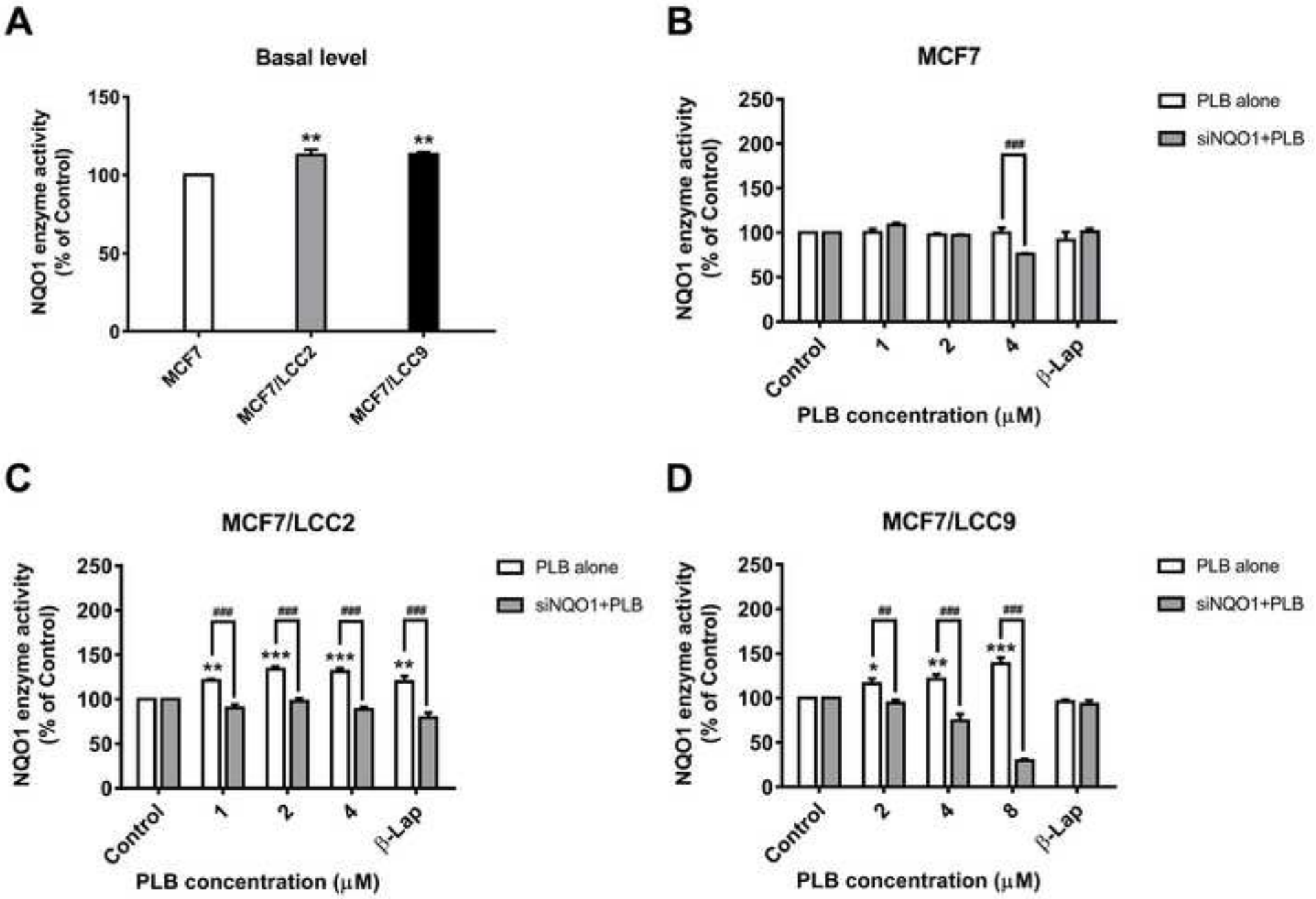

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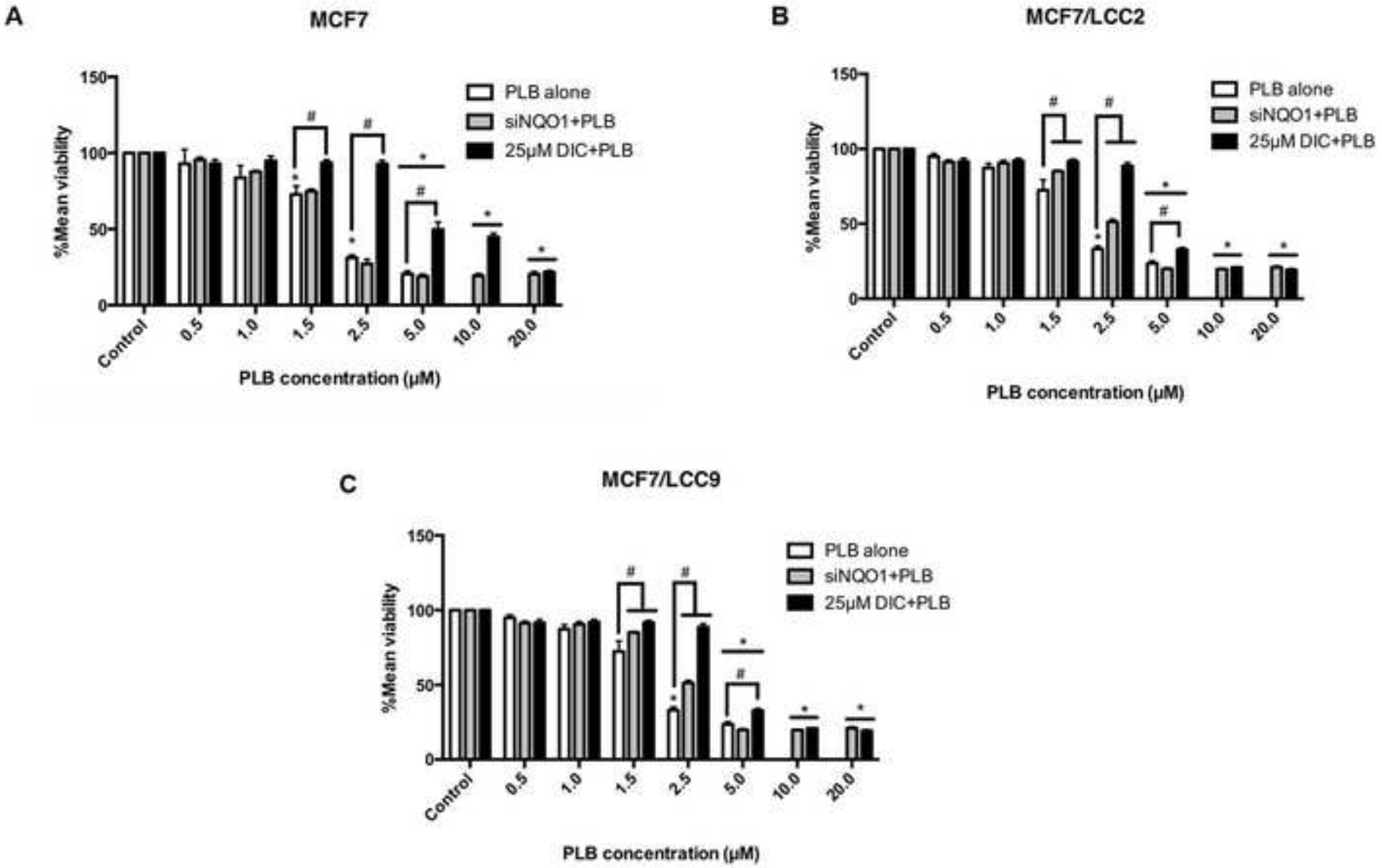

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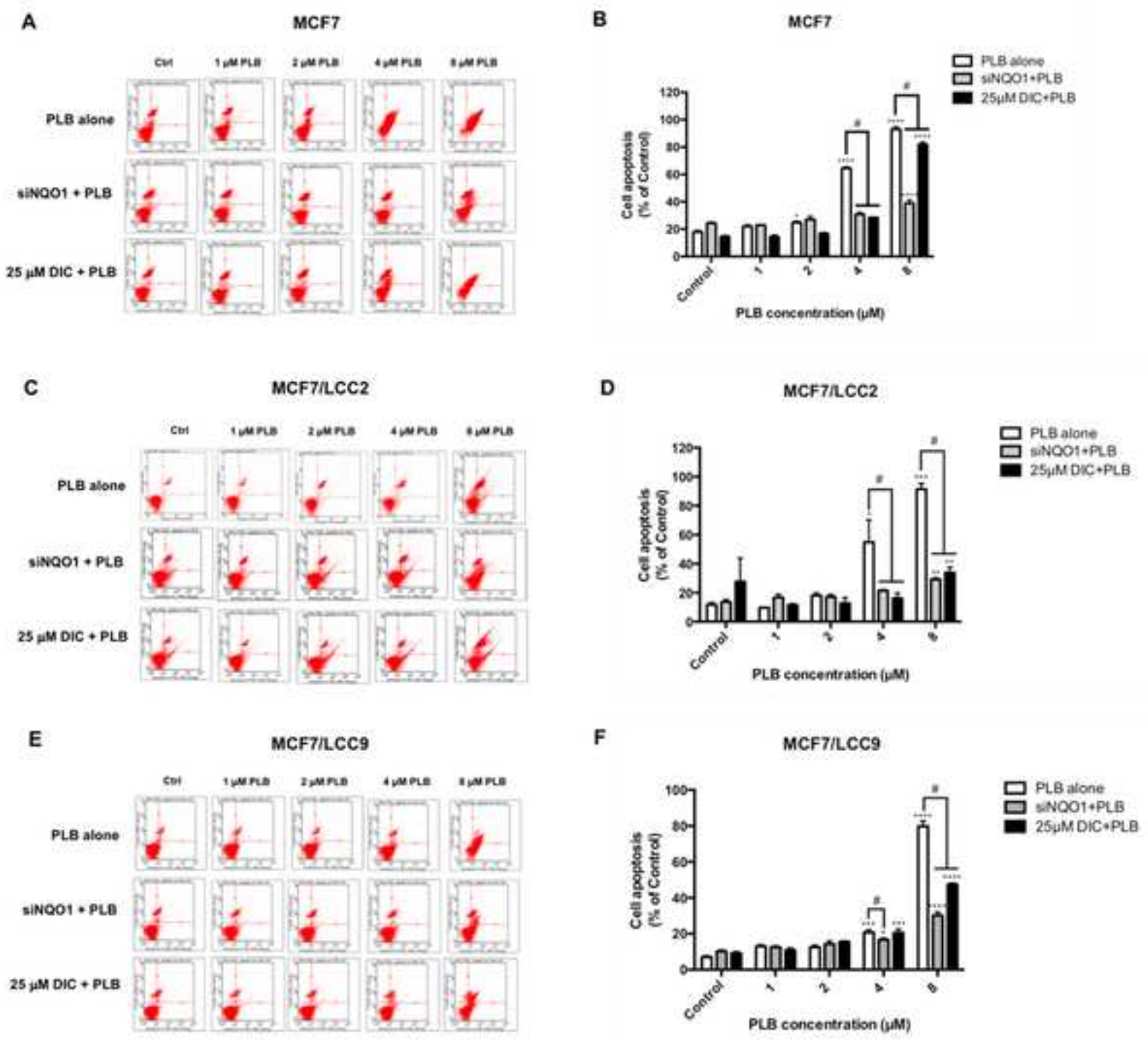

Figure5

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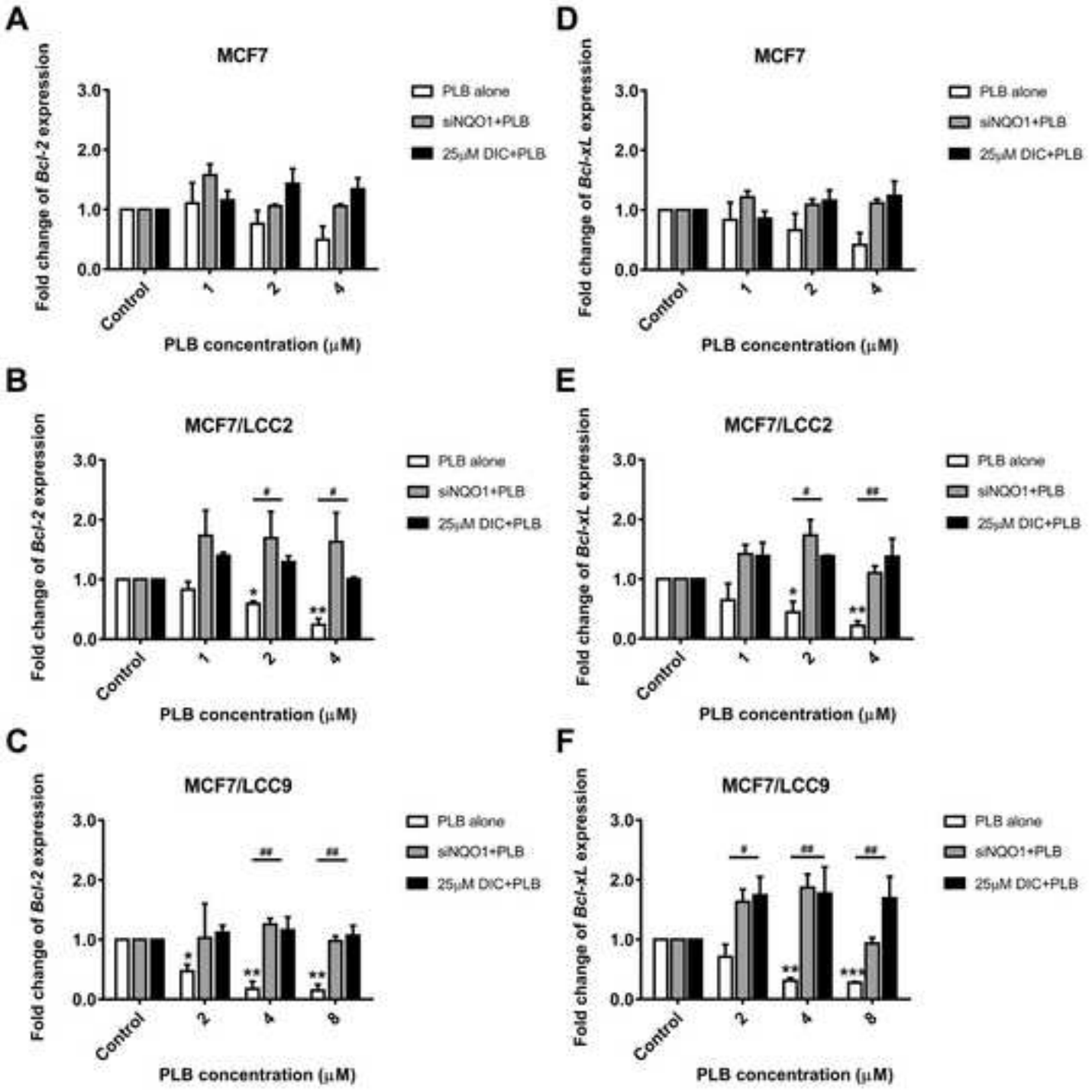

Figure6

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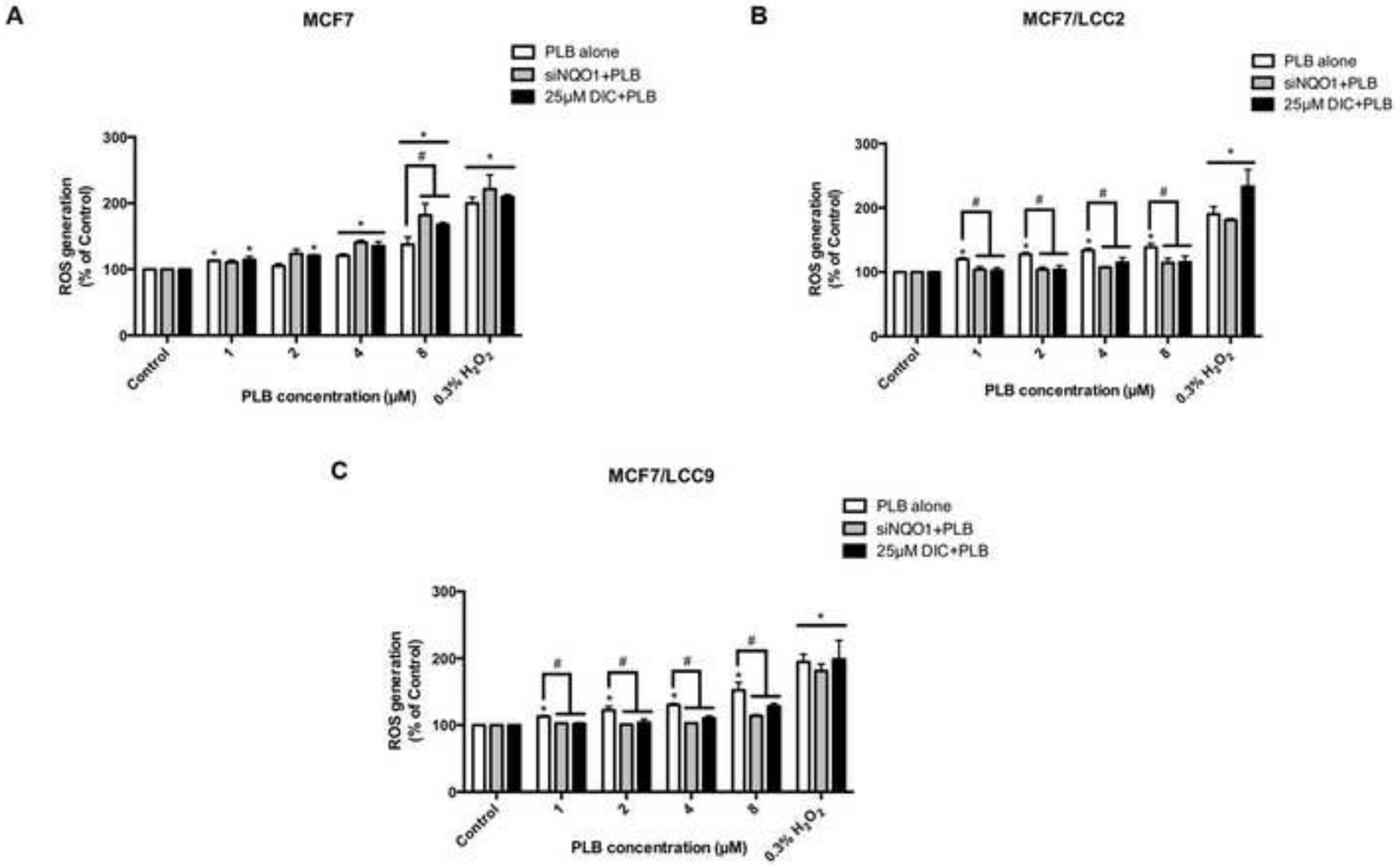

Figure7

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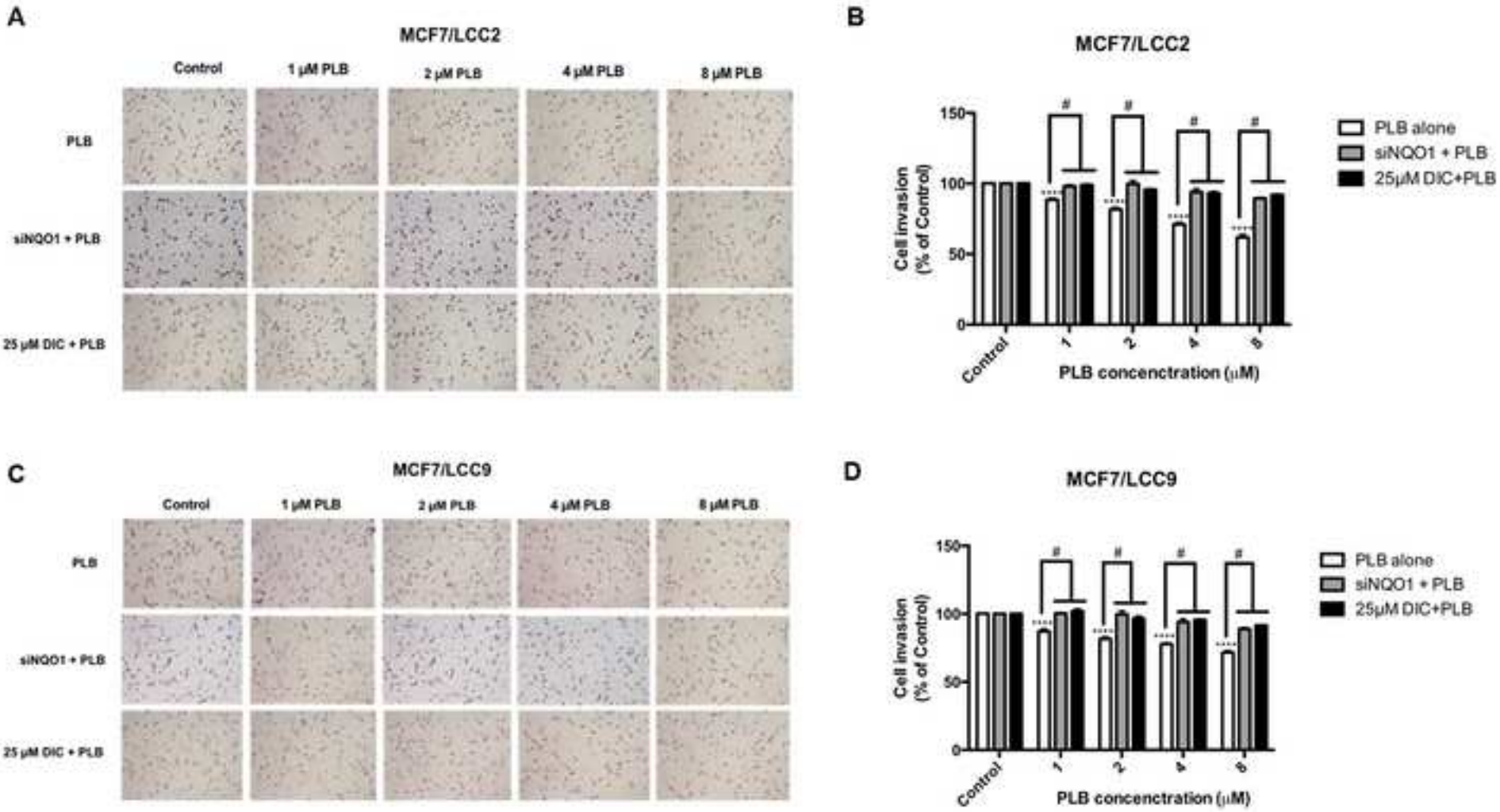

Figure8
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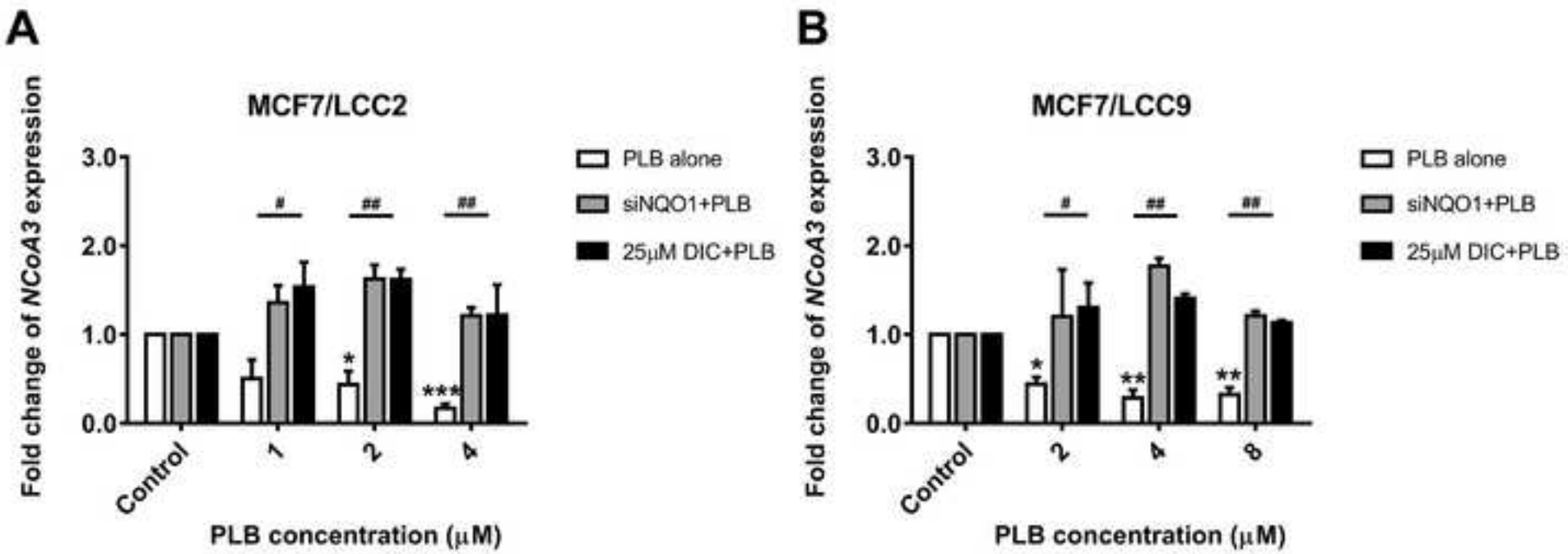
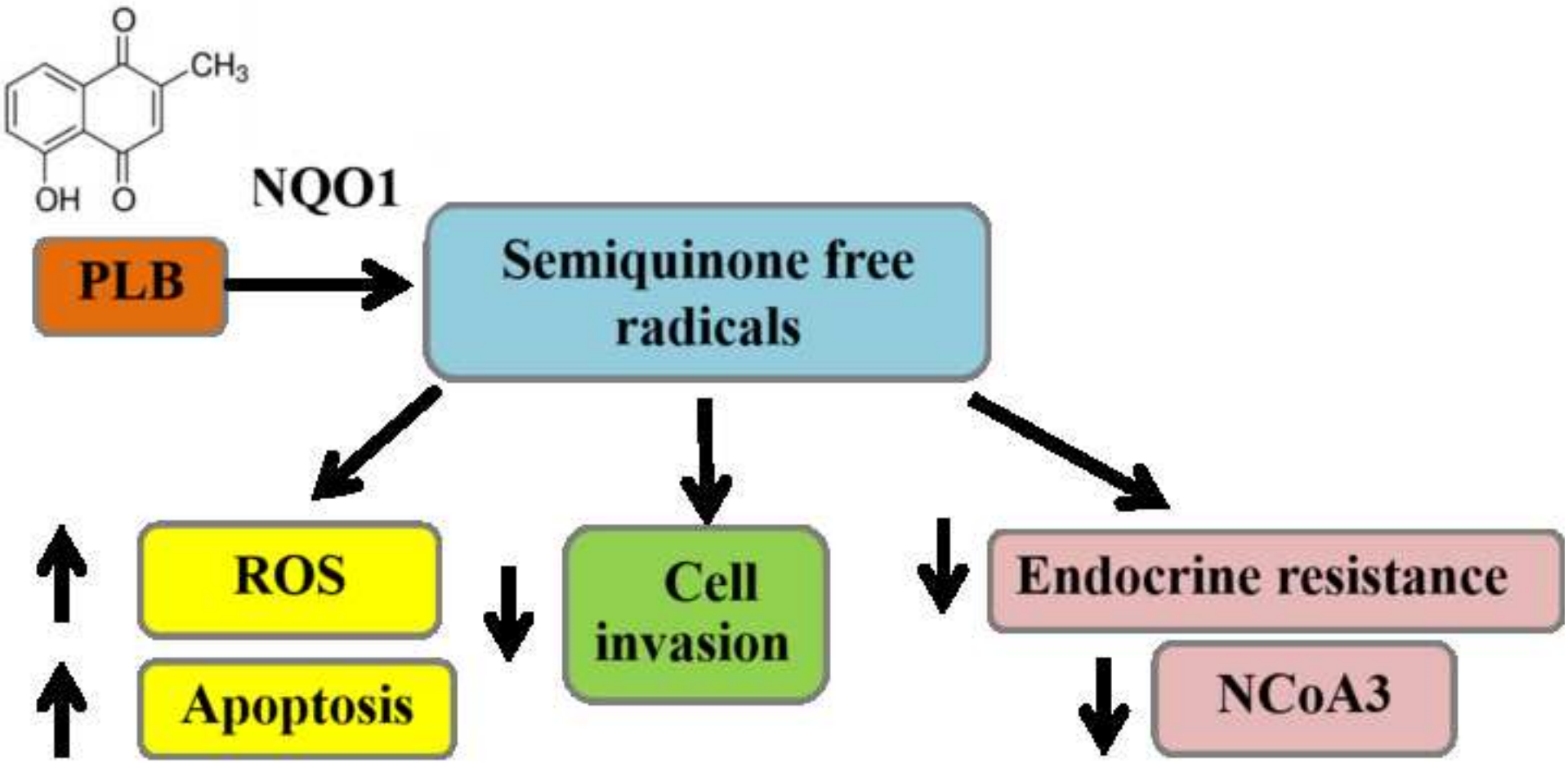
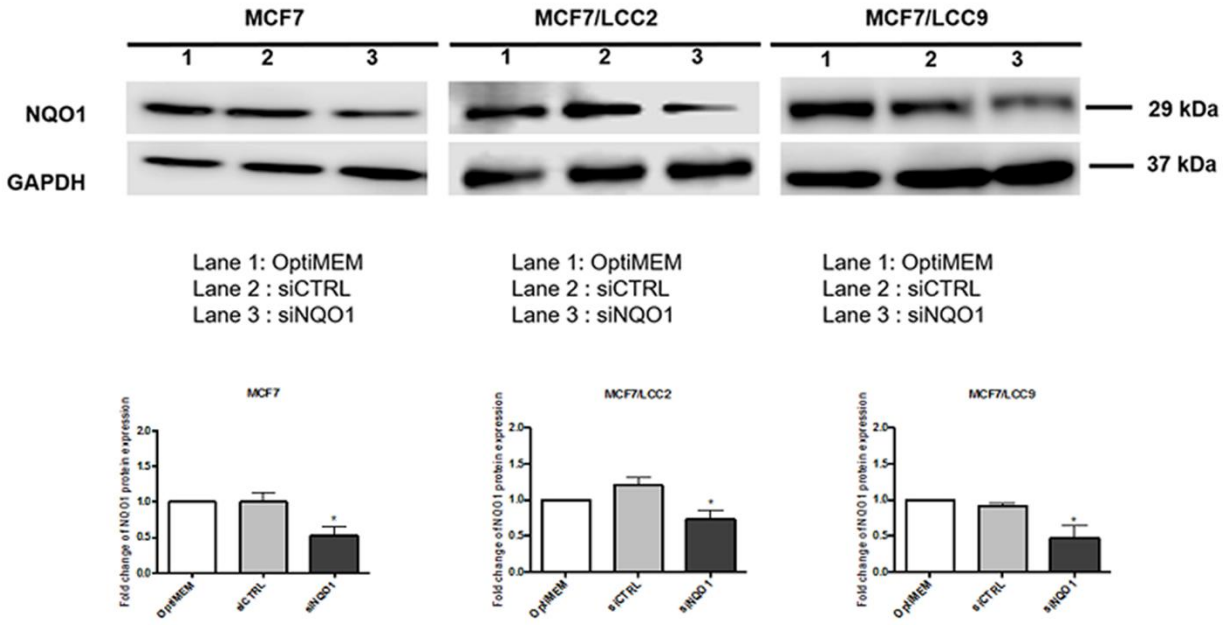


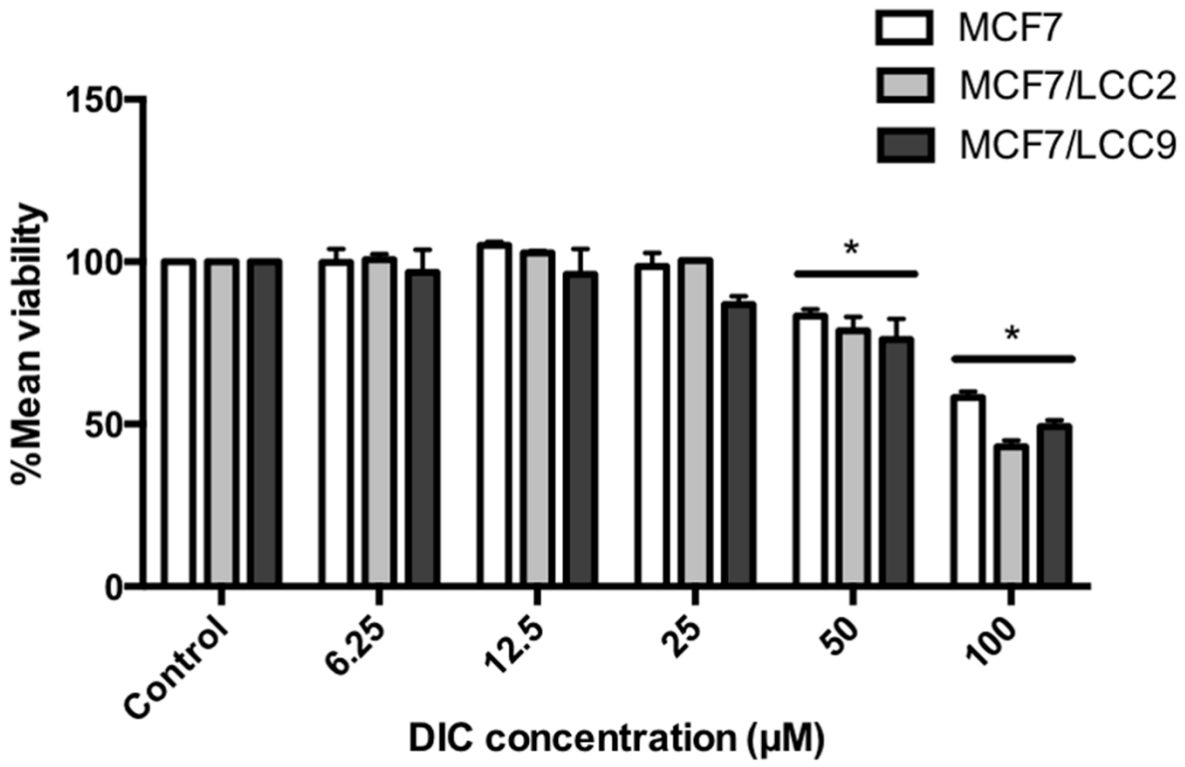
Graphical Abstract



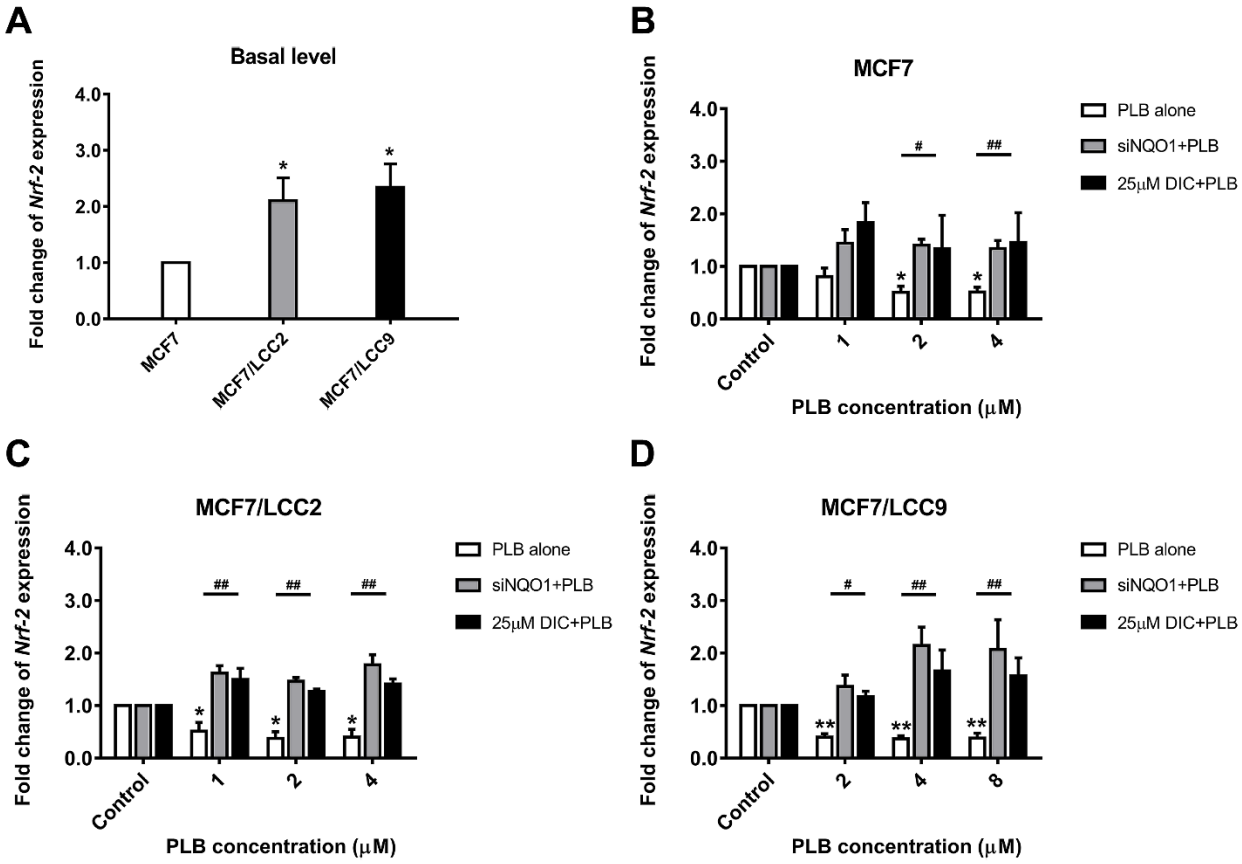
Supplementary figures



Supplementary figure 1. The level of NQO1 protein expression of the 3 cell lines after 24 hours of transfection with siNQO1 and negative control siRNA transfection using 1.5 µM of lipofectamine. The efficiency of the NQO1 knockdown by transient NQO1 siRNA transfection was confirmed by evaluating the levels of the protein expression. * represents $p < 0.05$ and ** represents $p < 0.01$ vs. siCTRL.



Supplementary figure 2. Dicoumarol (DIC) is NQO1 inhibitor and is not toxic to breast cancer cells. The bar chart illustrates mean percentage and standard error of the mean of cell viability of MCF7 cells against DIC at increasing concentrations after 48 h incubation (mean ± SEM, n=3). The IC_{50} of DIC on MCF7, MCF7/LCC2, and MCF7/LCC9 is >100 µM, 88.50 µM ± 3.40, and 98.76 µM ± 3.03, respectively. * represents $p < 0.05$ vs. control (0.2% DMSO).



Supplementary figure 3. **The inhibitory effect of PLB on *Nrf-2* expression was lower when the NQO1 was downregulated or its activity was inhibited**. (A) The baseline Nrf-2 levels for the 3 cell lines by qRT-PCR analysis (mean ± SEM, n=3). * represents $p < 0.05$ vs MCF7 cells. The *Nrf-2* expression for PLB alone, 25 μM DIC pretreatment, and 24 h after all 3 cell lines were transfected with siNQO1: MCF7 (B) MCF7/LCC2 (C) and MCF7/LCC9 (D) cells.

* represents $p < 0.05$, ** represents $p < 0.01$ vs. control (0.2% DMSO), and $^{\#}$ represents $p < 0.05$, $^{\#\#}$ represents $p < 0.01$ vs. PLB alone.