Characterization and Property Investigation of Novel Human Glucose 6-Phosphate Dehydrogenase Inhibitors in Lung Cancer Cell Lines

Miss Makamas Chanda

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การศึกษาลักษณะและคุณสมบัติของสารสกัดใหม่ต่อการยับยั้งเอนไซม์กลูโคส 6-ฟอสเฟต ดีไฮโดรจีเนส ในเซลล์มะเร็งปอดเพาะเลี้ยงของมนุษย์

น.ส.มาฆมาศ จันดา

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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Thesis Title	Characterization and Property Investigation of Novel Human Glucose 6-Phosphate Dehydrogenase Inhibitors in Lung Cancer Cell Lines
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มาฆมาศ จันดา : การศึกษาลักษณะและคุณสมบัติของสารสกัดใหม่ต่อการยับยั้งเอนไซม์กลูโคส 6-ฟอสเฟต ดีไฮโดรจีเนส ในเซลล์มะเร็งปอดเพาะเลี้ยงของมนุษย์ . (Characterization and Property Investigation of Novel Human Glucose 6-Phosphate Dehydrogenase Inhibitors in Lung Cancer Cell Lines) อ.ที่ปรึกษาหลัก : ผศ. ดร.ชาลิสา หลุยเจริญ ชีพสุนทร, อ.ที่ปรึกษาร่วม : รศ. ดร.พูลลาภ ชีพสุนทร

เอนไซม์กลูโคส 6-ฟอสเฟต ดีไฮโดรจีเนส (จีซิกพีดี) มีบทบาทสำคัญในกระบวนการต่างๆ ของเซลล์ รวมถึงการรักษาสมดุลของปฏิกิริยารีดอกซ์และการสังเคราะห์ไขมันและนิวคลีโอไทด์ มะเร็งหลายชนิดรวมถึงมะเร็งปอดถูกพบว่ามีระดับของเอนไซม์จีซิกพีดีที่เพิ่มสูงขึ้นเพื่อตอบสนองต่อการเพิ่มจำนวนและการอยู่รอดของเซลล์มะเร็ง ด้วยเหตุนี้การกำหนดเป้าหมายที่เอนไซม์จีซิกพีดีจึงถูกนำมาใช้เป็นกลยุทธ์ที่สำคัญในการยับยั้งการเจริญเติบโตของเซลล์มะเร็งในปัจจุบัน งานวิจัยในครั้งนี้จึงสนใจที่จะค้นหาสารสกัดจากธรรมชาติชนิดใหม่ที่มีคุณสมบัติไม่เพียงแต่ต้องยับยั้งการเจริญเติบโตของเซลล์มะเร็งเท่านั้นแต่ยังต้องมีคุณสมบัติในการยับยั้งการทำงานของเอนไซม์จีซิกพีดีได้อีกด้วย การศึกษาของเราได้ค้นพบว่าสารสกัดจากธรรมชาติ SJ006 ซึ่งเป็นสารอนุพันธ์ในกลุ่ม 1,2-แนพโทควิโนน สามารถยับยั้งการมีชีวิตของเซลล์มะเร็งปอดชนิดไม่เล็กได้และยังส่งผลในการยับยั้งโดยตรงที่การทำงานของเอนไซม์จีซิกพีดีโดยไม่รบกวนระดับเอ็มอาร์เอ็นเอและโปรตีน นอกจากนี้ยังพบว่า SJ006 มีคุณสมบัติในการเป็นตัวยับยั้งการทำงานของเอนไซม์จีซิกพีดีแบบไม่แข่งขันจากระดับของค่าสัมประสิทธิ์ของปฏิกิริยา (*Km*) และความเร็วสูงสุดของปฏิกิริยา (*Vmax*) ที่ลดลง ในการศึกษาครั้งนี้เรายังพบว่าการทดสอบด้วย SJ006 ส่งผลให้เกิดอนุมูลอิสระที่เพิ่มขึ้นจนทำให้เกิดการยับยั้งวัฏจักรการแบ่งตัวของเซลล์ที่ระยะ G2/M และกระตุ้นให้เซลล์มะเร็งปอดเกิดการตายแบบอะพอพโทซิสจากระดับของ *Bax/Bcl-2* ที่เพิ่มสูงขึ้น ยิ่งไปกว่านั้นการทดสอบด้วยดีไรโบส (D-(–)-ribose) สามารถเพิ่มจำนวนของเซลล์มะเร็งปอดจากการถูกยับยั้งด้วย SJ006 ได้ สนับสนุนบทบาทของ SJ006 ในการยับยั้งการเจริญเติบโตของเซลล์มะเร็งปอดผ่านทางวิถีเพนโทสฟอสเฟตที่เป็นผลมาจากการยับยั้งการทำงานของเอนไซม์จีซิกพีดี ในการศึกษาครั้งนี้จึงกล่าวได้ว่า SJ006 มีคุณสมบัติเป็นตัวยับยั้งการทำงานของเอนไซม์จีซิกพีดีชนิดใหม่แบบไม่แข่งขันที่มีฤทธิ์ในการต้านการเจริญเติบโตและการอยู่รอดของเซลล์มะเร็งปอดชนิดไม่เล็ก

สาขาวิชา ชีวเวชศาสตร์ (สหสาขาวิชา) ลายมือชื่อนิสิต .....

ปีการศึกษา 2565 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

5987788520 : MAJOR BIOMEDICAL SCIENCES (INTERDISCIPLINARY PROGRAM)
KEYWORD: G6PD; NSCLC; uncompetitive G6PD inhibitor; 12-naphthoquinone

Makamas Chanda : Characterization and Property Investigation of Novel Human Glucose 6-Phosphate Dehydrogenase Inhibitors in Lung Cancer Cell Lines . Advisor: Asst. Prof. Chalisa louicharoen Cheepsunthorn, Ph.D. Co-advisor: Assoc. Prof. POONLARP CHEEPSUNTHORN, Ph.D.

G6PD plays fundamental roles in many cellular processes, including redox balance and lipid and nucleotide synthesis. Overexpression of G6PD is required to promote the proliferation and survival of cancer cells, including lung cancer. Targeting G6PD is a prominent strategy to inhibit cancer cell progression. Finding novel natural compounds with anticancer properties to inhibit G6PD activity needs to be investigated. Our study indicated that SJ006, which is 1,2-naphthoquinone, promoted cytotoxic activity against NSCLC cell lines. Interestingly, SJ006 exhibited a direct inhibitory effect on G6PD activity without interfering with mRNA or protein levels, which were strongly observed in both A549 and H292. Additionally, an uncompetitive inhibition was also proposed as a property of SJ006 to inhibit G6PD in this study through the reduction of Km and Vmax. The increasing level of ROS production was observed, resulting in G2/M phase cell cycle arrest and the elevating of Bax/Bcl2 ratio through the inhibition of G6PD-induced apoptosis in NSCLC cell lines in the presence of SJ006. Moreover, D-(−)-ribose, a bypass product of the PPP, was able to rescue NSCLC proliferation in the presence of SJ006, supporting the inhibitory role of SJ006 on NSCLC proliferation through the G6PD-regulated PPP. Therefore, SJ006 was identified as a novel uncompetitive G6PD inhibitor with anticancer activity that inhibits the progression and survival of NSCLC.

Field of Study:	Biomedical Sciences (Interdisciplinary Program)	Student's Signature .....
Academic Year:	2022	Advisor's Signature .....
		Co-advisor's Signature .....

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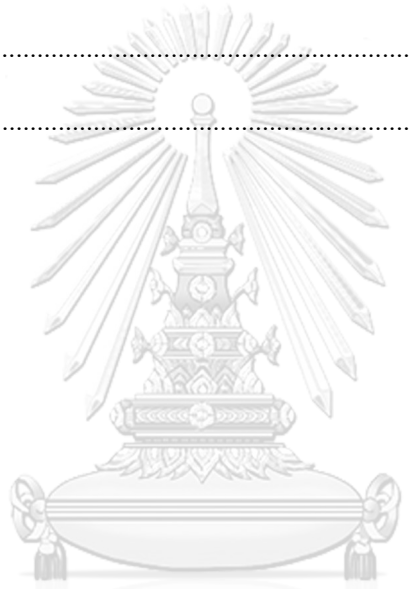

Section 1

1.1 The connection of all the articles presented in the dissertation.

This dissertation is written in manuscript form and consists of two manuscripts discussing the impact of glucose 6-phosphate dehydrogenase (G6PD) in non-small lung cancer (NSCLC) and the discovery of a novel G6PD inhibitor to suppress proliferation in NSCLC. G6PD is an enzyme that links glycolysis and the pentose phosphate pathway (PPP) using glucose 6-phosphate (G6P) to generate a coenzyme, nicotinamide adenine dinucleotide phosphate (NADPH). NADPH, mainly produced by G6PD, is required in various cellular processes, such as the antioxidant system and nucleotide and lipid synthesis. Overexpression of G6PD is considered one of the metabolic reprogramming mechanisms that promote cancer development and progression in several types of cancer, including lung cancer. Lung cancer, which is the most common cause of cancer-related death in both men and women worldwide, was chosen to explore the oncogenic role of G6PD in this study.

In the first manuscript, we demonstrated the significantly high expression level of G6PD in NSCLC tissues compared with those of small cell lung cancer (SCLC) tissues. These findings led us to further explore the function of G6PD in NSCLC progression. The results demonstrated a positive association between the expression level of G6PD and the NSCLC proliferation rate. Our studies verified the role of G6PD in NSCLC proliferation and migration by suppressing G6PD function with

dehydroepiandrosterone (DHEA) and G6PD small interfering RNA (siRNA). Moreover, inhibition of G6PD induced apoptosis in NSCLC cells. D-(-) ribose, a product of G6PD, was also added, confirming the oncogenesis of G6PD in NSCLC proliferation by rescuing the anti-proliferative effects of DHEA and G6PD siRNA. Our results supported the crucial role of G6PD in NSCLC proliferation and migration.

The second manuscript was conducted to investigate a novel G6PD inhibitor that not only restraining G6PD activity but also has the ability to suppress NSCLC progression. Moreover, Kirsten rat sarcoma viral oncogene homolog (KRAS) mutation is considered as a challenging therapeutic target, despite being "undruggable" in advanced NSCLC. Thus, targeting G6PD, an oncogene, may affect the survival of KRAS-mutated NSCLC. Dehydroepiandrosterone (DHEA) and 6-amino nicotinamide (6-AN) are currently used as G6PD inhibitors, but their use is limited by their non-specificity and side effects. Derivative compounds of ortho-naphthoquinones or 1,2-naphthoquinones (1,2-NQ) show a wide range of biological responses, including anticancer effects, and were selected as candidate compounds in this study. The cytotoxic concentration (CC_{50}) of derivative compounds of 1,2-NQ on NSCLC cells were examined. Interestingly, SJ006, 2-methyl-2,3-dihydronaphtho[1,2-b]furan-4,5-dione, is a derivative of 1,2-NQ and has a direct inhibitory effect on G6PD activity. SJ006 was discovered as a novel uncompetitive G6PD inhibitor by decreasing K_m and V_{max} according to the Michaelis-Menten equation. Moreover, reactive oxygen species (ROS) were markedly elevated in NSCLC cells as a result of the inhibitory effect of

SJ006 on the pentose phosphate pathway. Apoptosis and cell cycle arrest at the G2/M phase of SJ006-treated NSCLC cells increased in parallel with the decrease in the G0/G1 phase due to the ROS production. Our results indicated that SJ006, a compound with 1,2-NQ, exhibited the ability to block G6PD activity, thereby contributing to the inhibition of cancer proliferation and development in NSCLC cells.

All articles are part of the dissemination of the doctoral dissertation.

1.2 Background and rationale

The PPP is a metabolic pathway that runs parallel to glycolysis and serves multiple functions in cellular metabolism (1). One of its key roles is the generation of ribose 5-phosphate (R5P) for nucleotide synthesis and NADPH, a reducing equivalent necessary for biosynthetic processes and antioxidant defense mechanisms (2). G6PD is a critical enzyme within the PPP that catalyzes the first and rate-limiting step of the oxidative branch. G6PD has garnered significant attention due to its involvement in various aspects of tumor development and progression (1). Lung cancer cells often exhibit high levels of oxidative stress due to increased metabolic activity and ROS production (3, 4) Elevated G6PD activity helps cancer cells to counteract oxidative damage while promoting cell survival by producing NADPH to fulfill the higher demands (5, 6), and support the synthesis of nucleic acids, lipids, and membrane components necessary for cell growth and proliferation (2, 7). The evidence suggest that G6PD plays a significant role in cancer aggressiveness including metastasis in an

advanced stage of tumors (8), poor overall survival (9), and chemotherapy resistance (10).

Lung cancer is the most common cause of cancer related death worldwide in both men and women (11, 12, 13). Lung cancer can be classified into two main types of NSCLC and SCLC. NSCLC is the most common type, accounting for approximately 80-85% of all lung cancer cases (12, 14). Different three subtypes of NSCLC have been reported, including adenocarcinoma, which is the most prevalent subtype, squamous cell carcinoma, and large cell carcinoma. SCLC accounts for about 15% of lung cancer cases and tends to grow and spread more aggressively than NSCLC (15). Treatment approaches for lung cancer depend on the stage of the cancer, overall health of the patient, and specific molecular characteristics of the cancer. Surgery followed by adjuvant chemotherapy are the most favorable treatments and standard approaches for early- and advanced-stage lung cancer patients (stage II-IIIA) (16). Combination of a platinum-based regimen, like cisplatin, with pemetrexed or etoposide or paclitaxel is the first-line therapy commonly and currently used in the Thai guidelines in advanced NSCLC patients (17). However, resistance to chemotherapy and radiation therapy reduces treatment efficacy and hampers patient survival (10). In NSCLC, two significant molecular targets are the epidermal growth factor receptor (*EGFR*) and the Kirsten rat sarcoma viral oncogene homolog (*KRAS*), whose mutations induce cancer cell proliferation and survival (18). Targeted therapies are designed to specifically target certain genetic alterations or molecular

markers (*EGFR*, *KRAS)* in cancer cells. Gefitinib and erlotinib are the first generation of tyrosine kinase inhibitors (TKIs) that block the activity of mutated *EGFR* (19). KRAS belongs to a family of GTPases, in which the active state is regulated by guanosine triphosphate (GTP) and the inactive state is regulated by guanosine diphosphate (GDP) (20, 21). Unlike EGFR, KRAS was considered a challenging therapeutic target, despite being "undruggable" after drug targeting efforts over the past four decades (21, 22). The failure of *KRAS* mutation therapy is due to the difficulty in targeting the binding sites and their specificities for GTP (23, 24). Therefore, lung cancer patients with *KRAS* mutations had a worse prognosis and responded poorly to chemotherapy than those with *EGFR* mutations (25). Current research has attempted to target the *KRAS* mutation either directly through the development of novel KRAS inhibitors or indirectly through its role in various signaling pathways (24).

The overexpression of G6PD in lung cancer and the correlation between the expression level of G6PD and the aggressiveness parameters, such as chemotherapy resistant and poor prognosis of cancer have been reported (10, 26). Alterations in the PPP and G6PD have been associated with chemoresistance in lung cancer (cisplatin-resistant model) (10). The increasing of NADPH production by G6PD enhances antioxidant capacity in cancer cells, making them more resistant in the process of chemotherapy-induced oxidative stress (10). Additionally, the PPP provides building blocks for DNA and RNA synthesis, contributing to enhance mechanisms of DNA repair and resistance to DNA-damaging agents (27). Furthermore, poor survival was

significantly observed in lung cancer patients with G6PD-positive compared with patients with G6PD-negative (26). Our preliminary study found a significant overexpression of G6PD in lung cancers from the CU-DREAM analysis of NCBI's GEO dataset. However, the comparison of the expression level of G6PD in NSCLC and SCLC tissues has not been fully elucidated. Thus, the first study was aimed to investigate the association between the clinical characteristics and expression levels of G6PD in NSCLC and SCLC tissues. Moreover, the roles of G6PD in NSCLC proliferation and migration were investigated by blocking G6PD function using DHEA and G6PD siRNA. Our findings highlight that G6PD is a potential therapeutic target for the development of novel anti-cancer strategies in lung cancer therapy.

Targeting the G6PD in lung cancer therapy has been proposed as a strategy to disrupt redox balance, impair lipid synthesis, and sensitize cancer cells to oxidative stress and chemotherapy. DHEA, a non-competitive G6PD inhibitor, is a steroid hormone produced by the adrenal glands (28). The reduction in cancer cell proliferation, migration, xenograft formation, and induction of apoptosis has been demonstrated to be the anti-tumor effect of DHEA through inhibition of G6PD activity (28, 29). The mechanism of action of DHEA is still not fully understood because of its multifunctional hormonal properties (30). Some studies have disputed that the anticancer effects of DHEA were not related to G6PD inhibition but more likely due to mitochondrial gene suppression (31, 32). Moreover, high oral doses and the production of androgen via DHEA have been observed to be disadvantages of DHEA

(33). In addition, another known G6PD inhibitor is 6-AN, a competitive G6PD inhibitor that has been used in various tumors. By inhibiting the G6PD enzyme, 6-AN disrupts the production of NADPH and impairs the antioxidant defense mechanism of cancer cells. This leads to an accumulation of reactive oxygen species and can trigger cell death or sensitize cancer cells to chemotherapy or radiation therapy (10, 34). However, the non-specificity of 6-AN in inhibiting G6PD activity was reported because it competes instead with NADP+ (35) and 6-phosphogluconate dehydrogenase (6PGD) in PPP (36). This lack of specificity can lead to unintended consequences and side effects that may limit its therapeutic potential. Since G6PD exhibits a strong involvement in cell proliferation, migration, and the antioxidant system, finding a novel G6PD inhibitor to suppress G6PD activity in lung cancer cells is therefore interesting.

Quinone is an organic compound that is commonly found in animals, plants, and microorganisms (37). Several quinones, including naphthoquinones that contain naphthalene, have been demonstrated as sources of cytotoxic compounds with anticancer activity (38, 39). Doxorubicin, which contains a quinone moiety as part of its chemical structure, is a widely used chemotherapy drug that belongs to a class of anthracyclines (40). Ortho-naphthoquinones and 1,2-NQ has been investigated for its potential as an anticancer agent due to its cytotoxic properties (39, 41). The anticancer effect of a derivative compound of 1,2-NQ (ethoxy mansonone G) has been demonstrated as an anti-estrogenic property in estrogen receptor positive

breast cancer (42). Moreover, the anticancer activity of the 1,2-NQ derivative (butoxy mansonone G) has been suggested in NSCLC cell lines through the targeting of STAT3 and Akt signaling pathways (43). However, the anticancer property of 1,2-NQ in NSCLC via inhibiting G6PD activity, which disrupts the cancer cells' antioxidant defense mechanisms and impairs biosynthesis processes, has not been elucidated.

Taken together with the non-specific and limited uses of 6-AN and DHEA, the novel G6PD inhibitors with anticancer activities in NSCLC need to be examined. In this second study, we aimed to investigate the cytotoxic concentrations (CC_{50}) and inhibitory effects of derivative compounds of 1,2-NQ on G6PD activity in NSCLC cell lines. The candidate derivative compound of 1,2-NQ that exhibited a strong inhibitory effect on G6PD activity was used to verify the anticancer activity. Through blocking G6PD activity in NSCLC cells, the candidate 1,2-NQ compound was further investigated for its anti-proliferation, induction of oxidative stress, promotion of apoptosis, and activation of cell cycle arrest.

1.3 Objectives

The first manuscript aimed to

1. To investigate the expression level of G6PD in NSCLC and SCLC tissues and the clinical characteristics of NSCLC and SCLC patients
2. To demonstrate the role of G6PD in promoting the proliferation and migration of NSCLC cells using DHEA, G6PD siRNA, and D-(-)-ribose

The second manuscript aimed to

1. To investigate the properties of G6PD inhibition and the cytotoxic concentration (CC_{50}) of derivative compounds of 1,2-NQ in NSCLC cells
2. To examine the antitumor activity of a novel G6PD inhibitor containing 1,2-NQ by measuring the levels of cell proliferation, ROS, cell cycle arrest, and apoptotic marker expression in NSCLC cells

1.4 Scope of the research

Our first project focused on the role of G6PD in the proliferation of NSCLC cells by monitoring the correlation between the expression level of G6PD in NSCLC and SCLC tissues and clinical characteristics of NSCLC and SCLC patients. Moreover, the function of G6PD in promoting proliferation and migration in NSCLC cells was investigated. Promoting the proliferation and migration in NSCLC cells by G6PD was verified using G6PD inhibitor (DHEA), siRNA, and D-(-) ribose. The second project focused on the identification of novel G6PD inhibitors from 1,2-NQ-containing compound, which has anticancer properties in both *KRAS* mutant and *KRAS* wild-type NSCLC cells. The type of inhibitor, cell proliferation, cell cycle arrest, ROS levels, and apoptosis were examined.

1.5 Benefits of this research

Our study provides evidence to support the specificity of G6PD overexpression in NSCLC tissues and the positive correlation between the expression

level of G6PD and the proliferation of NSCLC cells. The study indicates that disruption of G6PD function affected NSCLC cell proliferation and migration. These findings support the effects of G6PD as a therapeutic target for NSCLC. This dissertation discovers SJ006 or 2-methyl-2,3-dihydronaphtho[1,2-b]furan-4,5-dione1,2-naphthoquinone compound, a novel uncompetitive G6PD inhibitor, that reduces K_m and V_{max}. This study provides new knowledge that SJ006 has an anticancer property by reducing cell proliferation, increasing ROS levels, promoting apoptosis, and activating cell cycle arrest at G2/M phase in the NSCLC model, in both *KRAS* mutant and *KRAS* wild-type NSCLC cell lines. Targeting G6PD using SJ006 may be a drug candidate for further study in the treatment of *KRAS* mutant NSCLC.

Section 2

2.1 First manuscript was written in the topic of

Inhibition of non-small cell lung cancer (NSCLC) proliferation through targeting G6PD

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Abstract (230 words)

Background: Mounting evidence has linked cancer metabolic reprogramming with altered redox homeostasis. The pentose phosphate pathway (PPP) is one of the energy metabolism-related pathways that has been enhanced to promote cancer growth. The glucose 6-phosphate dehydrogenase (G6PD) of this pathway generates reduced nicotinamide adenine dinucleotide phosphate (NADPH) essential for controlling cellular redox homeostasis. This research aimed to investigate growth promoting effects of G6PD in the non-small cell lung cancer (NSCLC).

Methods: Clinical characteristics and G6PD expression levels in lung tissues of 64 patients diagnosed with lung cancer at the King Chulalongkorn Memorial Hospital (Bangkok, Thailand) during 2009-2014 were analyzed. G6PD activity in NSCLC cell lines including NCI-H1975 and NCI-H292 was experimentally inhibited using DHEA and siG6PD to study cancer cell proliferation and migration.

Results: The expression of G6PD in lung cancer tissues was detected by immunohistochemical staining and was found to be associated with TNM stages. The association between position G6PD expression was significantly observed in NSCLC when compared to SCLC tissues. G6PD expression levels and activity also coincided with proliferation rate of NSCLC cell lines. Suppression of G6PD induced apoptosis in NSCLC cell lines by increasing Bax/Bcl-2 ratio expression. The addition of D-(-) ribose which is an end-product of the PPP increased the survival of G6PD-deficient NSCLC cell lines.

Discussion and Conclusion: Collectively, these findings demonstrated that G6PD might play an important role in the carcinogenesis of NSCLC. Therefore, G6PD could be used as a prognostic marker in NSCLC. Inhibition of G6PD might provide a therapeutic strategy for treatment of NSCLC.

Key words: G6PD**,** PPP, metabolic reprogramming, lung cancer, NSCLC

Introduction

Lung cancer accounts for the deaths of over 2 million people globally in 2018 (44). Non-small cell lung cancer (NSCLC) is the main type representing approximately 80 - 85% of lung cancer. If the cancer is detected early or before metastasis, the 5-year survival rate for NSCLC is predicted to be around 60% (45) Nonetheless, the average survival time drops to less than 12% without treatment (46). Small-cell lung

cancer (SCLC) is another less common but more aggressive type of lung cancer than NSCLC (15). It would be of benefit to have a better understanding of how lung cancer cells reprogram their metabolic pathways, since this knowledge may help in early diagnosis of lung cancer.

Energy metabolic reprogramming (EMR) and altered redox homeostasis are emerging hallmarks of cancer (47, 48). In addition to aerobic glycolysis or Warburg effect, the pentose phosphate pathway (PPP), a branch of glycolysis, is crucial for tumorigenesis (47). PPP is composed of the oxidative branch and the non-oxidative branch (49). The oxidative branch, in which glucose 6-phosphate dehydrogenase (G6PD) is a rate-limiting enzyme, generates reduced nicotinamide adenine dinucleotide phosphate (NADPH) and ribose 5-phosphate (R5P), which are necessary for controlling redox homeostasis, biosynthesis of fatty acids and cholesterol, and serving as a key component in the synthesis of nucleotides (2, 50, 51). On the other hand, the non-oxidative branch produces fructose 6-phosphare (F6P), glyceraldehyde 3-phosphare (G3P), and pentose phosphates, which are supplements for glycolysis and anabolic pathways (49). Upregulation of G6PD has been reported to associate with metastasis, advanced stage, and poor overall survival time (2, 50, 51) in many malignancies including colorectal cancer (52), bladder cancer (53), breast cancer (8, 50), clear cell renal cell carcinoma (9) and lung cancer (10). Lower survival rate was observed in lung cancer with G6PD-positive compared to G6PD-deficiency patients (26).

In this study, we aimed to examine the expression of G6PD in lung tissues from patients diagnosed with lung cancer. We also aimed to elucidate growth promoting effects of G6PD in NSCLC cell lines.

Materials and methods

Tissue specimens

This study was approved by the Institute Ethics Committee of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand (IRB 561/59). The study protocol was performed according to the declaration of Helsinki for the participation of human individuals. Lung tissue specimens were obtained from 64 lung cancer patients, who admitted to the King Chulalongkorn Memorial Hospital (Bangkok, Thailand) during 2009-2014. All tissue samples were examined by a team of pathologists at the King Chulalongkorn Memorial Hospital to determine the type and stage of cancer. Clinical data were collected at the time of first diagnosis and continued until recurrence, death, or the last follow-up appointment.

Histological and immunohistochemical analyses

Formalin-fixed and paraffin-embedded lung tissue blocks were serially cut into 5 μm thick sections, mounted on slides in serial order and processed following standard procedures for histological evaluation. Hematoxylin and Eosin (H&E) staining was performed to determine the cancerous and adjacent non-cancerous areas on

the sections. To detect G6PD protein expression, tissue sections adjacent (serial) to H&E-stained sections were heated in an autoclave 120 °C for 10 min for antigen retrieval, treated with hydrogen peroxide to quench endogenous peroxidase and blocked with corresponding serum from a secondary antibody raised. Subsequently, the sections were incubated with anti-G6PD antibody produced in rabbit (HPA000247; Sigma-Aldrich, St. Louis, MO, USA) at a dilution of 1:1000 as manufacturer's recommendation at 4 °C overnight. Detection was performed using a biotinylated secondary antibody (Sigma-Aldrich) followed by a streptavidin-biotin complex peroxidase (1:200; Vector, Burlingame, CA, USA) and visualized with 3,3'-diaminobenzidine tetrahydrochloride (Sigma-Aldrich). An isotype IgG antibody was used as a negative control. No counterstain was used. The stained sections were evaluated by two pathologists who were blinded to the patient's clinical information. The G6PD H-score was calculated using the formula 1×(% of weakly-stained as light brown, 1+) +2×(% of moderately stained as medium brown, 2+) +3×(% of strongly stained as dark brown, 3+), as described previously (54).

Cell culture and treatments

Human NSCLC cell lines, NCI-H1975 (lung epithelial cells derived from adenocarcinoma tissue; ATCC# CRL-5908) and NCI-H292 (a lymph node metastasis of a pulmonary mucoepidermoid carcinoma; ATCC# CRL-1848), were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Both cell lines were

cultured and routinely passaged in RPMI-1640 medium containing 10% heat inactivated FBS and 1% Penicillin/Streptomycin solution (Merck Millipore, MA, USA) at 37°C in 5% CO_2.

Dehydroepiandrosterone (DHEA; Sigma-Aldrich) was prepared as a 1000-fold stock solution in dimethyl sulfoxide (DMSO), thus a final concentration of DMSO did not exceed 0.1%. D-(-)-Ribose (R9629; Sigma-Aldrich) was dissolved in culture medium and sterilized by filtration through 0.22 μm filter before use. After treatment with DHEA for 48 h, the cells were used as indicated in each experiment.

siG6PDs and transfection assay

Cells were plated in 12-well plated at 30-50% confluency in complete medium 24 h before transfection. Transfection was performed using Lipofectamine® 3000 (ThermoFisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. The siG6PDs for G6PD (sense sequence: GGCCGUCACCAAGAACAUU) and non-silencing scramble (sense sequence: GGCACUACCAGACACGAUU) were synthesized and purified by Integrated DNA Technologies, Inc., (IDT, Coralville, IA. USA) and were used at 100 nM final concentration. After transfection of siG6PDs for 24 h, the cells were used in the indicated experiments.

Cell proliferation assay

After treatment, cells were incubated with MTT tetrazolium salt (final concentration 0.5 mg/ml, Sigma-Aldrich) for 2 h at 37 °C. The formazan crystal product was then dissolved with DMSO and the optical absorbance was measured at 570 nm using a Synergy HT microplate reader (BioTek instruments Inc., Winooski, VT, USA).

Colony forming assay

Cells were seeded in 6-well plate at a density of $2x10^2$ cells/well 24 h before beginning treatment. After treatment the culture medium was removed and replaced with fresh complete medium. The cells were then continuously cultured for 7 days. At the end, the colonies were washed with 1X PBS, fixed with glutaraldehyde (0.6% v/v), stained with crystal violet (0.5% w/v) for 30 min and counted.

G6PD activity assay

After treatments, the cell pellets were collected at the indicated times, washed with ice-cold PBS. The cells were resuspended with cold PBS, sonicated for 10 seconds (repeated three times) and cooled on ice. Total protein was determined by BCA protein assay kit (ThermoFisher Scientific, USA), according to the manufacturer's instructions. G6PD enzyme activity was measured as previously described (55) with some modification. Briefly, 20 μl of the cell lysate (protein at

1mg/ml concentration) was mixed with 980 μl of the reaction buffer, containing 0.38 mM NADP, 6.3 mM $MgCl_2$, 3.3 mM glucose 6-phosphate, and 5 mM maleimide in 50 mM Tris-HCl (pH 7.5) buffer. The absorbance was kinetically measured at 340 nm for 15 min at 37°C using a Synergy HT microplate reader (BioTek instruments Inc., USA). Enzyme activity was calculated using a standard curve of NADPH and expressed as NADPH unit per min per mg of total protein.

Scratch wound assay

Cells were seeded in 24-well plates to a final density of 1 x 10^5 cells/well and maintained in CO_2 incubator at 37ºC for 24 h to allow cell adhesion. The confluent monolayer was scratched with a sterile 200-μl pipette tip. Then, culture medium containing dislodged cells was immediately removed and replaced with fresh medium, either alone or containing DHEA or siG6PDs. The scratched areas were monitored by collecting digitized images at various time points or until closure of the wound in control monolayer.

Quantitative RT-PCR

Total RNA was isolated from cell pellets using TRIzol® Reagent (ThermoFisher, USA), according to the manufacturer's instructions. The quality and concentration of RNA was determined using NanoDrop 2000 spectrophotometer (ThermoFisher Scientific, USA). cDNAs were synthesized using a RevertAid First Strand

cDNA Synthesis Kit (ThermoFisher Scientific, USA), according to the manufacturer's instructions. Gene expression analysis was performed using PowerUp™ SYBR® Green Master Mix (Thermo Fisher Sciencetific, USA) on a StepOnePlus real-time PCR machine (Thermo Fisher Sciencetific, USA), according to manufacturer's protocol. Primer sequences were as follows: G6PD forward primer 5'-GTC AAGGTGTTGAAATGCATC-3' and reverse primer 5'-CATCCCACCTCTCATTCTCC-3', Bax forward primer 5'-AACATGGAGCTGCAGAGGAT-3' and reverse primer 5'- CAGC CCATGATGGTTCTGAT-3', Bcl2 forward primer 5'-GGTGGGGTCATGTGTGTG-3' and reverse primer 5'- CGGTTCAGGTACTCAGTCATC-3', and ß-actin as a reference gene forward primer 5'-ACTCTTCCAGCCTTCCTTC-3' and reverse primer 5'- ATCTCCTTC TGCATCCTGTC-3'. The relative abundance of each target gene was calculated relative to ß-actin. The fold change in expression levels was reported as $2^{-\Delta\Delta Ct}$.

Statistical analysis

Statistical analyses were performed using SPSS v.22.0 (SPSS, Inc., Chicago, IL, USA). Data were obtained from three independent experiments performed in triplicate and were presented as the mean ± standard deviation (SD). Chi-Square test (χ^2) and Student's *t*-test were used to examine the differences of categorical and quantitative variables. A two-sided difference with a *p*-value less than 0.05 was considered statistically significant.

Results

Characteristics of patients

A total of 64 lung cancer patients consisting of 44 NSCLC patients (68.75%) and 20 SCLC patients (31.25%) were examined in this study. Demographic data of the subjects are summarized in **Table 1.** The mean age of the patients was 69.5±12.45 years, ranging from 30 to 94 years. Clinical profiles of NSCLC patients were adenocarcinoma 63.64% (28/44) and squamous cell carcinoma 36.36% (16/44). Most of lung adenocarcinoma patients were female (19/28, 67.86%). Male patients were diagnosed with lung squamous cell carcinoma more than females (15/16, 93.75%). Stage at the time of diagnosis was determined according to the tumor, node and metastasis (TNM) staging system, as shown in **Table 1**. There were three lung cancer patients (one from each category: adenocarcinoma, squamous cell carcinoma and SCLC) that we were unable to obtain their TNM stages. In comparing with NSCLCs, most of SCLC patients had significantly poor prognosis due to factors such tumor size (6.3±3.1 cm.) (p=0.009), lymph node metastasis (85% of all SCLC cases) (p<0.001), distant metastasis (75% of all SCLC cases) (p<0.001), and late stage (III-IV) (94.74% of all SCLC cases) (p<0.001).

Table 1. Summary of patient characteristics

Clinical parameters	Total n=64 (%)	NSCLC n=44 (%)	SCLC n=20 (%)	*p*-value*	NSCLC		
					Adeno-carcinoma n=28 (%)	Squamous cell carcinoma n=16 (%)	*p*-value#
Males	40 (62.5)	24 (54.5)	16 (80.0)	0.051	**9 (32.1)**	**15 (93.8)**	**<0.001**
Age (Years) (Mean±SD)	69.5±12.5	70.1±13.2	68.2±10.8	0.577	68.2±11.4	73.4±15.8	0.208
Tumor size (cm.) (Mean±SD)	4.6±2.6	**4.0±2.1**	**6.3±3.1**	**0.009**	**3.2±1.2**	**5.3±2.7**	**0.001**
Lymph node metastasis	33 (51.6)	**16 (36.4)**	**17 (85.0)**	**<0.001**	11 (39.3)	5 (31.3)	0.594
Distant metastasis	29 (45.3)	**14 (31.8)**	**15 (75.0)**	**0.001**	11 (39.3)	3 (18.8)	0.172
TNM							
Early stage (I, II)	61 (95.3)	27 (64.29)	1 (5.26)	**<0.001**	19 (70.37)	8 (53.33)	0.270
Advance stage (III, IV)		15 (35.71)	18 (94.74)		8 (29.63)	7 (46.67)	
G6PD IHC positive	38 (59.3)	**36 (81.8)**	**2 (10.0)**	**<0.001**	24 (85.7)	12 (75.0)	0.434$
H-score (Mean±SD)	40.7±31.9	**41.5±32.6**	**25.8±1.1**	**0.007**	**30.3±23.2**	**63.9±37.9**	**0.013**
Recurrent	17 (26.6)	13 (29.5)	4 (20.0)	0.315	9 (32.1)	4 (25.0)	0.171

* Compared between NSCLC & SCLC

Compared between adenocarcinoma and squamous cell

$ Fisher's exact test

G6PD immunostaining

To examine whether G6PD expression could be correlated with the tumorigenesis of lung tissues, we performed an immunohistochemistry of G6PD

protein in lung tissues obtained from lung cancer patients. Results revealed that G6PD protein was highly expressed in cancerous areas of lung tissues. The staining was more intense in NSCLC than in SCLC tissues (p<0.001) (**Figure 1**). Consistently, the H-scores of G6PD expression were higher in NSCLC than in SCLC tissues (p=0.007) (**Table 1**). Among NSCLC tissues, the H-scores of G6PD in squamous cell carcinoma were higher than that of in adenocarcinoma tissues, (p=0.013) (**Table 1**). Results also indicated that positive G6PD expression was positively correlated with TNM stages, especially in advance stage of lung cancer (stage III-IV) (p=0.019).

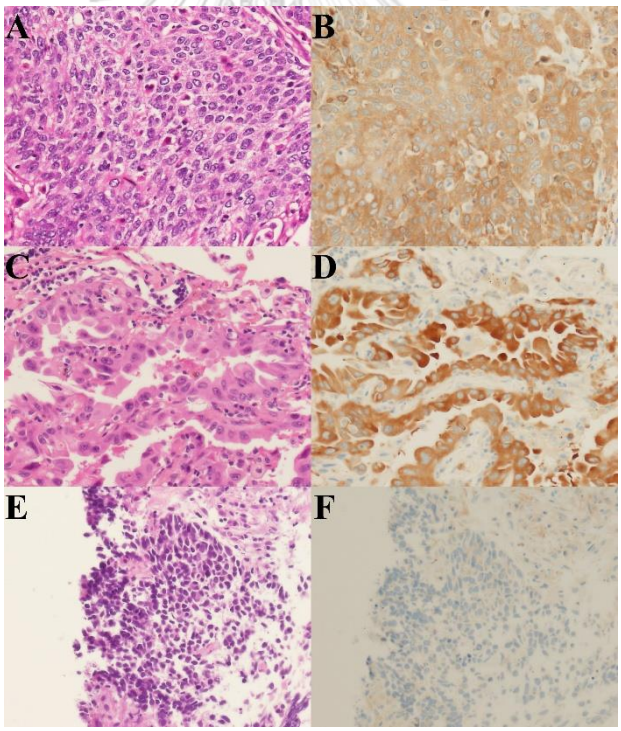


Figure 1. Representative images of H&E staining (left column) and G6PD IHC staining (right column) of lung cancer tissues from patients with (A, B) squamous cell carcinoma, (C, D) adenocarcinoma, and (E, F) SCLC. (200x magnification)

G6PD and NSCLC cell proliferation

In this set of experiments, we aimed to investigate a correlation between G6PD expression and NSCLC growth and advancement. The growth of two NSCLC cell lines with different characteristics were compared. The first cell line was NCI-H292 cell and the other cell line was NCI-H1975 cell. The former was derived from lymph node metastasis of a pulmonary mucoepidermoid carcinoma, whereas the latter were lung epithelial cells derived from adenocarcinoma tissue. Both cells were seeded at the same cell density. After 48 hours, the MTT cell proliferation assay was performed to quantify the number of viable cells. Results showed that NCI-H292 cells had a higher proliferation rate than NCI-H1975 cells (**Figure2A**). Then, we compared mRNA expression levels and activity of G6PD in both cell lines. Results showed that mRNA expression levels and activity of G6PD were significantly higher in NCI-H292 cells than in NCI-H1975 cells (**Figure2, B and C**). These findings suggested that expression levels and activity of G6PD had a positive correlation with growth and advancement of NSCLC cells.

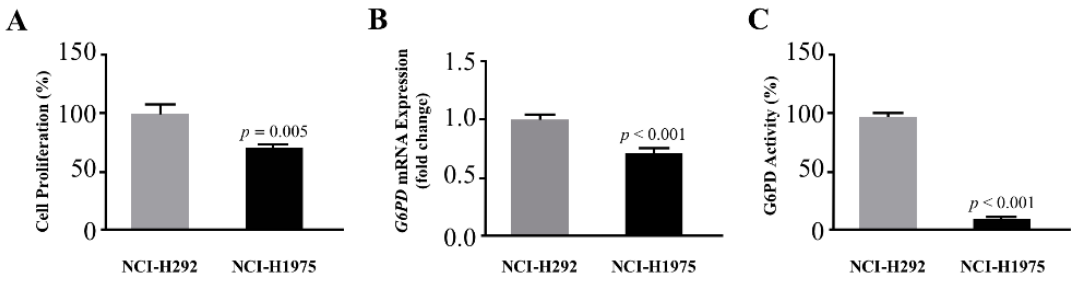


Figure 2. Comparison of (A) the MTT cell proliferation, (B) G6PD mRNA expression levels, and (C) G6PD activity in NCI-H292 and NCI-1975 cells at 48 hours after seeding at the same cell density.

Inhibition of G6PD reduced NSCLC cell proliferation.

We challenged the role of G6PD for growth and advancement of NSCLS cells by using DHEA, a well-known G6PD inhibitor, and small interfering RNA (siRNA). Results in the first set of experiments with DHEA demonstrated that, after 48 hours of treatment, DHEA exhibited a concentration-dependent manner in reduction of G6PD activity in both NCI-H292 and NCI-H1975 cells (**Figure 3, A and B**). Although DHEA at 400 μM showed superior results in reduction of G6PD activity in both NSCLC cells, as compared to DHEA at 300 μM, we observed its incomplete solubility at this concentration. Therefore, we selected DHEA at the concentration of 300 μM for the evaluation of NSCLC cell proliferation using MTT and colony forming assays. After 48 hours of treatment, DHEA significantly reduced cell proliferation of both NSCLC cells (**Figure 3C**). If NSCLC cells were allowed to grow in normal culture conditions for 7 days after DHEA treatment, there were no colonies observed at the end of the experiment compared to corresponding controls (**Figure 3D**).

In the next set of experiments, siG6PD was used to suppress G6PD expression in NSCLC cells. The efficacy of siG6PD after 24 hours of application was evaluated by measuring G6PD mRNA levels and G6PD activity. Results showed that siG6PD

significantly reduced G6PD expression and G6PD activity in NCI-H1975 and NCI-H292 cells by more than 50% compared to scramble controls (**Figure 4 A and B**). After 24 hours of siG6PD application, cell proliferation of NSCLC cells was determined. Results showed that siG6PD significantly reduced cell proliferation of both NCI-H1975 cells and NCI-H292 cells by approximately 20%, compared to scramble controls (**Figure 4C**).

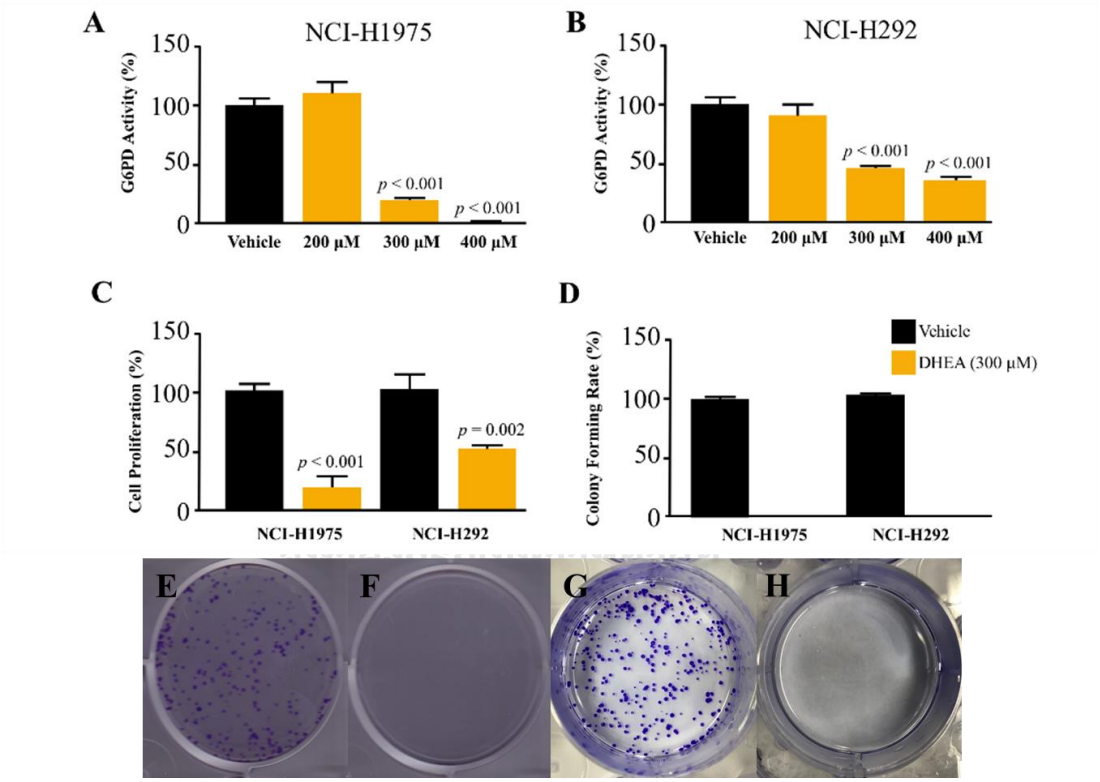


Figure 3. Inhibitory effects of DHEA on (A, B) G6PD activity and (C) cell proliferation in NCI-H1975 cells and NCI-H292 cells at 48 hours. (D) Colony formation in NCI-H1975 and NCI-H292 at day 7 after 48 hours of DHEA treatment.

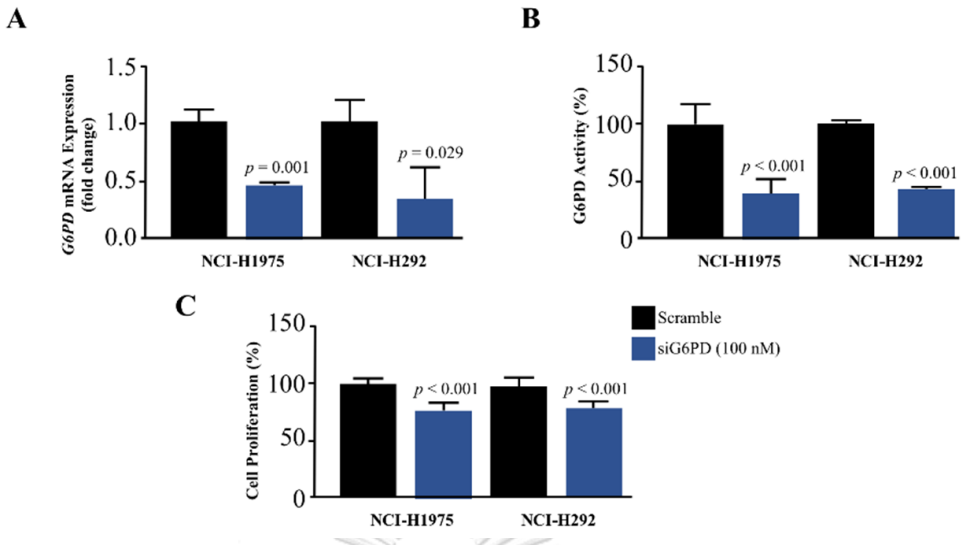
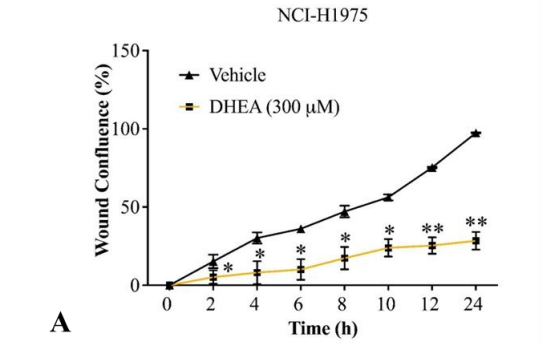
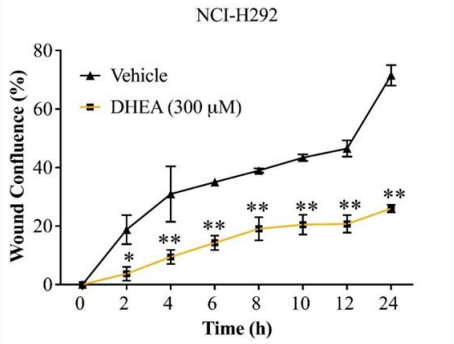


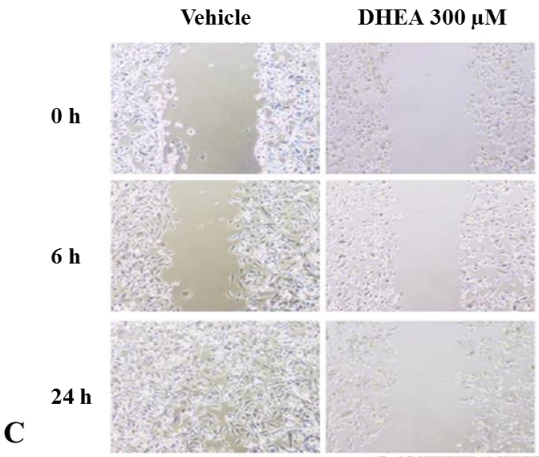
Figure 4. Inhibitory effects of siG6PD after 24 hours of application on (A) G6PD mRNA levels, (B) G6PD activity, and (C) cell proliferation of NCI-H1975 and NCI-H292 cells.

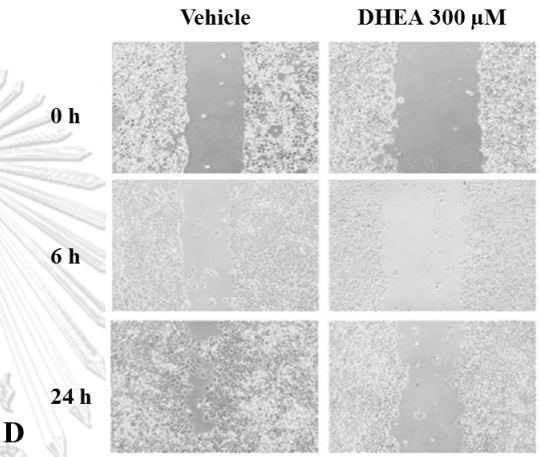
Inhibition of G6PD affected NSCLC cell migration.

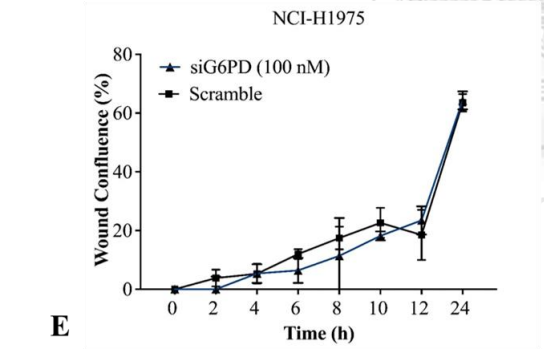
In this set of experiments, a scratch-wound assay was performed 24 hours post-treatment to assess the effects of G6PD inhibition on the contribution of NSCLC cell migration to wound closure. Results demonstrated that DHEA at 300 μM decreased wound confluence by increasing unmigrated area in cultures of NCI-H1975 and NCI-H292 cells, compared with that of the untreated controls at every time points examined (**Figure 5A-D**). Initially, an application of siG6PD slightly decreased wound confluence in cultures of NCI-H292 cells, compared with that of scramble controls. However, its effect was significantly observed at 24 hours post-application (**Figure 5F and H**). In contrast, an application of siG6PD showed less effective in decreasing wound confluence in cultures of NCI-H1975 cells at all time points examined, when compared to scramble controls (**Figure 5E and G**).

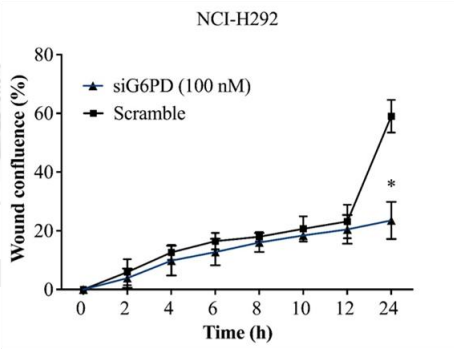

NCI-H1975
Vehicle
DHEA (300 μM)
Wound Confluence (%)
Time (h)
A

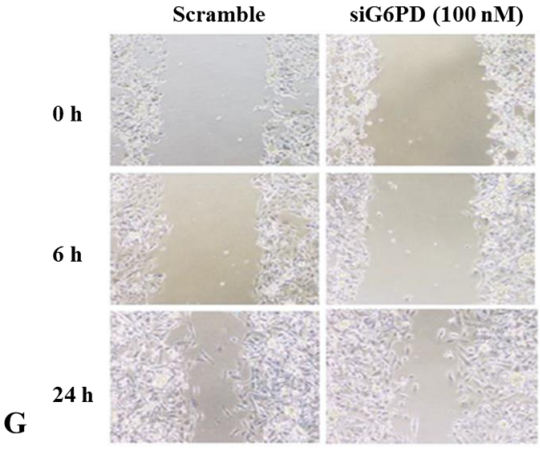

NCI-H292
Vehicle
DHEA (300 μM)
Wound Confluence (%)
Time (h)
B


Vehicle
DHEA 300 μM
0 h
6 h
24 h
C


Vehicle
DHEA 300 μM
0 h
6 h
24 h
D


NCI-H1975
siG6PD (100 nM)
Scramble
Wound Confluence (%)
Time (h)
E


NCI-H292
siG6PD (100 nM)
Scramble
Wound confluence (%)
Time (h)
F


Scramble
siG6PD (100 nM)
0 h
6 h
24 h
G

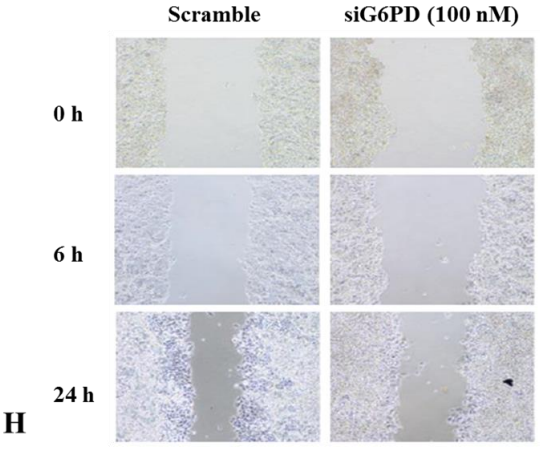

Scramble
siG6PD (100 nM)
0 h
6 h
24 h
H

Figure 5. Inhibitory effects of DHEA and siG6PD on wound confluence of NSCLC cells in a scratch-wound assay over 24 hours period as indicated. Percentage of wound confluence (A) and representative images of the assay (C) in cultures of NCI-H1975 cells and of NCI-H292 cells (B and D) in the presence of 300 μM DHEA, compared to that of corresponding vehicle controls. Percentage of wound confluence (E) and representative images of the assay (G) in cultures of NCI-H1975 cells and of NCI-H292 cells (F and H) after siG6PD application, compared to that of corresponding scramble controls.

DHEA and siG6PD induced NSCLC cell apoptosis.

To investigate whether mechanism underlying inhibitory effects of G6PD on NSCLC cell proliferation involved the induction of apoptosis, we measured the levels of Bax/Bcl2 mRNA ratio. Results indicated that the reduction of G6PD activity after 48 hours of DHEA treatment and down-regulation of G6PD expression by siG6PD after 24 hours post-application significantly induced apoptosis in NCI-H292 cells by increasing Bax/Bcl2 mRNA ratio, when compared to corresponding controls. However, the same treatments did not induce significant changes in Bax/Bcl2 mRNA ratio in NCI-H1975 cells (**Figure 6**).

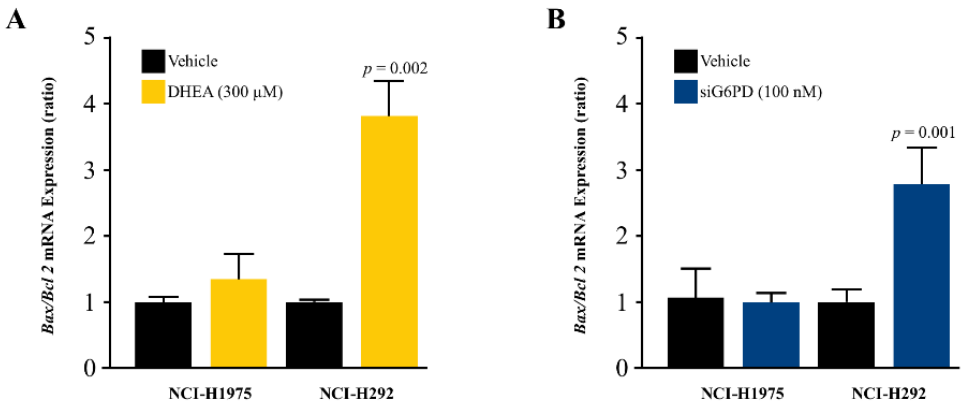


Figure 6. Effects of (A) DHEA at 48 hours post-treatment and (B) siG6PD at 24 hours post-application on the levels of Bax/Bcl-2 mRNA ratio in NCI-H1975 and of NCI-H292 cells.

Co-treatment effects of D-(-)-ribose and DHEA/siG6PD on NSCLC cell proliferation

To highlight the role of PPP in NSCLC cell proliferation, we asked whether the addition of D-(-)-ribose, an end-product of PPP, could reverse the inhibitory effects of DHEA and siG6PD on NSCLC cell proliferation. In this set of experiments D-(-)-ribose was co-administered with either DHEA or siG6PD. Results demonstrated that addition of D-(-)-ribose did not alter cell proliferation of NCI-H1975 cells that were treated with DHEA or siG6PD, compared to DHEA or siG6PD treated controls (**Figure 7A and B**). In contrast, D-(-)-ribose increased cell proliferation of NCI-H292 cells treated with DHEA or siG6PD to reach the levels of untreated control cells in a concentration dependent manner (**Figure 7C and D**).

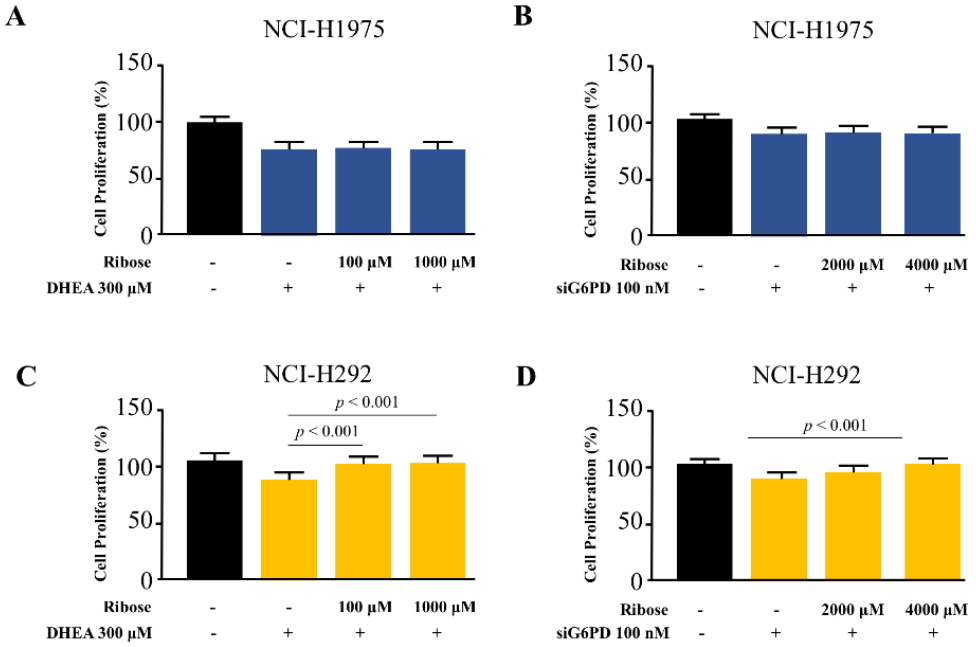


Figure 7. Effects of Co-treatment of D-(-)-ribose and DHEA/siG6PD on NSCLC cell proliferation. MTT assay was performed to evaluate cell proliferation of NCI-H1975 cells (A and B) and NCI-H292 cells (C and D) that were co-treated with D-(-)-ribose and DHEA for 48 hours or D-(-)-ribose and siG6PD for 24 hours.

Discussion and conclusion

G6PD is the rate-limiting enzyme in PPP and has been linked to tumorigenesis of many types of cancer, including lung cancer. However, its expression in prognostic-stages of lung cancer and the role of G6PD activity in NSCLC cell proliferation has not been completely elucidated. Here, we reported here a positive correlation between high expression levels of G6PD and late stage of lung cancer patients. Furthermore,

the results from a series of *in vitro* experiments indicated the important role of G6PD in NSCLC cell proliferation.

Most of lung cancer patients in this study were elderly with a mean age of 69.5±12.4 years similar to other study (56). This can be explained by a number of risk factors including delayed diagnosis, erroneous staging, lack effective screening method, and patient's behaviors including smoking habit and prolonged exposure to carcinogen and duration of carcinogenesis (57, 58). NSCLC was found to be the most common subtype. In agreement with previous report, male patients in this study were more likely to be affected by squamous cell carcinoma (58, 59). On the other hand, our female patients had a larger frequency of adenocarcinoma.

This present study reported for the first time that G6PD was highly expressed in NSCLC tissues, compared to SCLC tissues. G6PD expression was more intense in squamous cell carcinoma than adenocarcinoma. This finding was in line with CU-DREAM analysis of NCBI's GEO dataset program, targeting the overexpression of G6PD protein in lung cancer tissues compared to adjacent normal tissues (60). Our study further demonstrated a positive correlation between the advance stages of NSCLC and G6PD overexpression consistent with previous report of high levels of G6PD expression in poor prognosis lung cancer patients (26). These findings have implications for targeting the role of G6PD in carcinogenesis and the development of novel strategies for lung cancer therapy.

According to the American Type Culture Collection (ATCC) cell line information, NCI-H292 cell line originates from lymph node metastasis sites, while NCI-H1975 cell line originates from the primary tumor site. This information is in line with our findings of high levels of G6PD activity and mRNA expression in NCI-H292 cell line compared to that of NCI-H1975 cell line. The differences in G6PD levels in these two NSCLC cell lines positively correlated with their cell proliferation rates, suggesting the importance of G6PD in growth and advancement of NSCLC cells.

Our findings that DHEA treatment and application of siG6PD reduced cell proliferation and colony formation of NSCLS cells were supported by previous studies. Fang et al (2016) showed that DHEA and shRNA decreased the viability of cervical cancer cells (29). DHEA treatment suppressed the colony formations of hepatocarcinoma and breast cancer cells (61, 62). Therefore, targeting G6PD might be of therapeutic benefit for several types of cancer.

The present study demonstrated DHEA treatment and siG6PD application reduced NSCLC cell migration in a scratch-wound assay. These findings were supported by several studies in cervical cancer (29), oral squamous cell carcinoma (63) and breast cancer (62). The underlying mechanisms might involve the suppression of epithelial-mesenchymal transition (EMT) through E-cadherin activation (63). Additionally, we demonstrated that inhibition of G6PD activity and down-regulation of G6PD mRNA expression elevated the Bax/Bcl2 mRNA ratio, indicating an

increasing susceptibility of NSCLC cells to apoptosis. DHEA and siG6PD could deplete two main products of G6PD, NADPH and ribose via the PPP. Low levels of NADPH could increase the susceptibility of NSCLC to oxidative stress. When G6PD was inhibited, ROS and redox imbalance could occur, leading to ROS-mediated apoptosis (64). Simultaneously, reduced levels of ribose could compromise DNA synthesis in NSCLC cells, leading to cell cycle arrest and apoptosis respectively. These mechanisms could be used to explain the reduction in wound confluence in cultures of NSCLC cells treated with DHEA and siG6PD.

In order to study the proliferative effect in combination with DHEA or siG6PD on G6PD inhibition, D-(−)-ribose, the precursor for nucleic acid synthesis, has been used as a supplement. In G6PD repressed NSCLCs, D-(−)-ribose reversed the antiproliferative effects for the first time, as shown in our study. However, NCI-H292 was able to re-proliferate at low concentrations of D-(−)-ribose after inhibited by DHEA whereas high concentration was needed in the inhibition of G6PD mRNA via siG6PD. As mentioned in NCI-H1975, previous study also demonstrated the incapability of D-(−)-ribose to rescue the growth of G6PD knockdown cells possibly due to the insufficient level of G6PD on ROS-scavenging and ribose synthesis under oxidative stressed condition or DNA damage (65). Different concentrations of D-(−)-ribose have been used in various cancer cell types differently (66, 67), possibly due to the nature, aggressiveness and baseline G6PD activity level in each lung cancer cells. As stated in previous study, differential regulation of metabolic pathway in

different NSCLC subtypes may contribute to different metabolic vulnerabilities which can be indicated as potential therapeutic targets. (68), Therefore, G6PD could be a potential therapeutic target for NCI-H292 cell lines. Taken together, these can be stated that suppression of G6PD by DHEA or siG6PD altered EMR through the regulation of PPP in lung cancer cells lines.

Altogether, our findings demonstrated for the first time that G6PD is predominantly expressed in NSCLC and clarified the key roles of G6PD in lung cancer cells proliferation and apoptosis through the regulation of PPP and nucleotides synthesis. Therefore, G6PD could be a potential therapeutic strategy for lung cancer treatment.

Abbreviations

G6PD: Glucose 6-phosphate dehydrogenase

NSCLC: Non-small cell lung cancer

SCLC: Small cell lung cancer

DHEA: Dehydroepiandrosterone

siG6PD: Small interfering glucose 6-phosphate dehydrogenase

NADPH: Nicotinamide adenine dinucleotide

EMR: Energy metabolic reprograming

PPP: Pentose phosphate pathway

Ethics approval and consent to participate

This study was approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University (COA No. 1034/2016 IRB No.561/59). The protocol of this study was performed according to the Declaration of Helsinki for the participation of human individuals.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

CLC designed the research study. KR collected samples and performed the IHC. MC and PA performed cell culture research. MC and PA analyzed and interpreted the data and drafted the manuscript. CLC and PC revised the manuscript. All authors read and approved the final manuscript.

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2.2 Second manuscript was written in the topic of

Identification of 1,2-naphthoquinone as a novel uncompetitive human glucose 6-phosphate dehydrogenase inhibitor in lung cancer cell lines

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Abstract (164 words)

Overexpression of G6PD promotes lung cancer proliferation and survival by generating NADPH, which is essential for maintaining intracellular redox homeostasis as well as the synthesis of lipids and nucleotides. Targeting G6PD through the discovery of G6PD inhibitors is a prominent strategy to inhibit cancer cell progression. Our study indicated that SJ006 (2-Methyl-2,3-dihydronaphtho[1,2-b] furan-4,5-dione), which is 1,2-naphthoquinone, promoted cytotoxic activity against non-small cell lung cancer (NSCLC) cell lines. Interestingly, SJ006, a natural compound extracted from *Lichen Usnea* (Old Man's Beard), could inhibit the activity of G6PD, which reduced both the K_m and the V_{max} of the enzyme. Blocking G6PD activity induced ROS production, which consequently promoted G2/M phase cell cycle arrest and apoptosis by elevating the Bax/Bcl_2 ratio in NSCLC. Moreover, D-(−)-ribose, a product of the pentose phosphate pathway (PPP), was able to rescue NSCLC proliferation in the presence of SJ006, suggesting the role for SJ006 in inhibiting NSCLC proliferation through G6PD-regulated PPP. Hence, SJ006 (1,2-naphthoquinone) was identified as a novel uncompetitive G6PD inhibitor with anticancer activity that inhibits the progression and survival of NSCLC.

Key words: G6PD**,** NSCLC, uncompetitive G6PD inhibitor, 1,2-naphthoquinone

Introduction

The pentose phosphate (PPP) pathway is a metabolic pathway that operates in parallel with glycolysis and performs many functions in cellular metabolism. The important role of PPP is the generation of ribose 5-phosphate (R5P) for the synthesis of nucleotides and nicotinamide adenine dinucleotide phosphate (NADPH), which is an essential reducing agent for biosynthetic processes and antioxidant defense mechanisms. G6PD is a critical enzyme within the PPP that catalyzes the first and rate-limiting step of the oxidative branch. G6PD has garnered significant attention due to its involvement in various aspects of tumor development and progression (1). Cancer cells, including lung cancer cells, often exhibit high levels of oxidative stress due to increased metabolic activity and reactive oxygen species (ROS) production (Ref). Elevated G6PD activity in cancer cells helps meet the increased demand for NADPH, allowing cancer cells to counteract oxidative damage, promote cell survival (5, 6), and support the synthesis of nucleic acids, lipids, and membrane components necessary for cell growth and proliferation (7-13) (7). The evidence suggest that G6PD plays a significant role in cancer aggressiveness including metastasis in an advanced stage of tumors (8), poor overall survival (9), and chemotherapy resistance (10).

Lung cancer is the most common cause of cancer related death worldwide in both men and women (11, 12, 13). Poor prognosis has been observed in lung cancer patients, with a 5-year survival rate less than 18% and over half of lung cancer

patients die within one year (12). There are two main types of lung cancer: NSCLC and SCLC. NSCLC is the most common type of lung cancer, accounting for approximately 80-85% of cases (12, 14). Treatment approaches for lung cancer depend on the stage of the cancer, overall health of the patient, and specific molecular characteristics of the cancer. Surgery followed by adjuvant chemotherapy are the most favorable treatments and standard approaches for early- and advanced-stage lung cancer patients (stage II-IIIA) (16). Combination of a platinum-based regimen, like cisplatin, with pemetrexed or etoposide or paclitaxel is the first-line therapy commonly and currently used in the Thai guidelines in advanced NSCLC patients (17). However, resistance to chemotherapy and radiation therapy reduces treatment efficacy and hampers patient survival (10). In NSCLC, two significant molecular targets are the epidermal growth factor receptor (*EGFR*) and the Kirsten rat sarcoma viral oncogene homolog (*KRAS*) (18), whose mutations induce cancer cell proliferation and survival. Targeted therapies are designed to specifically target certain genetic alterations or molecular markers (*EGFR*, *KRAS)* in cancer cells. Gefitinib and erlotinib are the first generation of tyrosine kinase inhibitors (TKIs) that block the activity of mutated *EGFR* (19). KRAS belongs to a family of GTPases, in which the active state is regulated by guanosine triphosphate (GTP) and the inactive state is regulated by guanosine diphosphate (GDP) (20, 21). Unlike EGFR, KRAS was considered a challenging therapeutic target, despite being "undruggable" after drug targeting efforts over the past four decades (21, 22). The failure of *KRAS* mutation

therapy is due to the difficulty in targeting the binding sites and their specificities for GTP (23, 24). Therefore, lung cancer patients with *KRAS* mutations had a worse prognosis and responded poorly to chemotherapy than those with *EGFR* mutations (25). (18, 69, 70). Current research has attempted to target the *KRAS* mutation either directly through the development of novel KRAS inhibitors or indirectly through its role in various signaling pathways (24).

The overexpression of G6PD in lung cancer and the correlation between the expression level of G6PD and the aggressiveness parameters, such as chemotherapy resistant and poor prognosis of cancer have been reported (10, 26). Alterations in the PPP and G6PD have been associated with chemoresistance in lung cancer (cisplatin-resistant model) (10). The elevated NADPH production by G6PD can enhance the antioxidant capacity of cancer cells, making them more resistant to chemotherapy-induced oxidative stress. Additionally, the PPP provides building blocks for DNA and RNA synthesis, contributing to enhanced DNA repair mechanisms and resistance to DNA-damaging agents. Furthermore, poor survival was significantly observed in lung cancer patients with G6PD-positive compared with patients with G6PD-negative (26). Our study found a significant overexpression of G6PD in lung cancers from the CU-DREAM analysis of NCBI's GEO dataset. Our immunohistochemistry demonstrated a greater G6PD overexpression in NSCLC lung cancer tissues compared to small cell lung cancer (SCLC). Moreover, the overexpression of G6PD was strongly observed in cisplatin-resistant lung cancer cell

lines corresponded with its relapse (10, 71). Our findings highlight that G6PD is a potential therapeutic target for the development of novel anti-cancer strategies in lung cancer therapy.

Targeting the G6PD in lung cancer therapy has been proposed as a strategy to disrupt redox balance, impair lipid synthesis, and sensitize cancer cells to oxidative stress and chemotherapy. Dehydroepiandrosterone (DHEA) and aminonicotinamide (6-AN) are inhibitors commonly used against G6PD activity in various tumors, respectively (28, 72, 73). DHEA, a non-competitive G6PD inhibitor, is a steroid hormone produced by the adrenal glands (28). The reduction in cancer cell proliferation, migration, xenograft formation, and induction of apoptosis has been demonstrated to be the anti-tumor effect of DHEA through inhibition of G6PD activity (28, 29). The mechanism of action of DHEA is still not fully understood because of its multifunctional hormonal properties (30). Some studies have disputed that the anticancer effects of DHEA were not related to G6PD inhibition but more likely due to mitochondrial gene suppression (31, 32). Moreover, high oral doses and the production of androgen via DHEA have been observed to be disadvantages of DHEA (33). In addition, another known G6PD inhibitor is 6-AN, a competitive G6PD inhibitor that has been used in various tumors. By inhibiting the G6PD enzyme, 6-AN disrupts the production of NADPH and impairs the antioxidant defense mechanism of cancer cells. This leads to an accumulation of reactive oxygen species and can trigger cell death or sensitize cancer cells to chemotherapy or radiation therapy (10, 73) (5, 34,

74). However, the non-specificity of 6-AN in inhibiting G6PD activity was reported because it competes instead with NADP+ (35) and 6-phosphogluconate dehydrogenase (6PGD) in PPP (36). This lack of specificity can lead to unintended consequences and side effects that may limit its therapeutic potential. Since G6PD exhibits a strong involvement in cell proliferation, migration, and the antioxidant system, finding a novel G6PD inhibitor to suppress G6PD activity in lung cancer cells is therefore interesting.

Quinone is an organic compound that is commonly found in animals, plants, and microorganisms (37). Several quinones, including naphthoquinones (NQ) that contain naphthalene, have been demonstrated as sources of cytotoxic compounds with anticancer activity (38, 39). Doxorubicin, which contains a quinone moiety as part of its chemical structure, is a widely used chemotherapy drug that belongs to a class of anthracyclines (40). Ortho-naphthoquinones and 1,2-naphthoquinone (1,2-NQ) has been investigated for its potential as an anticancer agent due to its cytotoxic properties (39, 41). The anticancer effect of a derivative compound of 1,2-NQ (ethoxy mansonone G) has been demonstrated as an anti-estrogenic property in estrogen receptor positive breast cancer (42). Moreover, the anticancer activity of the 1,2-NQ derivative (butoxy mansonone G) has been suggested in NSCLC cell lines through the targeting of STAT3 and Akt signaling pathways (43). However, the anticancer property of 1,2-NQ in NSCLC via inhibiting G6PD activity, which disrupts the cancer cells'

antioxidant defense mechanisms and impairs biosynthesis processes, has not been elucidated.

Taken together with the non-specific and limited uses of 6-AN and DHEA, the novel G6PD inhibitors with anticancer activities in NSCLC need to be examined. In this study, we aimed to investigate the cytotoxic concentrations (CC_{50}) and inhibitory effects of derivative compounds of 1,2-NQ on G6PD activity in NSCLC cell lines. The candidate derivative compound of 1,2-NQ that exhibited a strong inhibitory effect on G6PD activity was used to verify the anticancer activity. Through blocking G6PD activity in NSCLC cells, the candidate 1,2-NQ compound was further investigated for its anti-proliferation, induction of oxidative stress, promotion of apoptosis, and activation of cell cycle arrest. SJ006 exhibited a strong inhibitory effect on G6PD activity, which promotes anti-proliferative and apoptotic effects in NSCLC. By considering their anti-cancer activities, 1,2-NQ may act as a good prominent inhibitory substance for G6PD in NSCLC.

Materials and method

Cells lines

Human lung cancer cell lines: NCI-H292 (ATCC# CRL-1848) (KRAS and EGFR wild type) and A549 (ATCC# CCL-185) (KRAS mutant and EGFR wild type) were maintained in Dulbecco's Modified Eagle's Medium (DMEM, HyClone, Logan, UT, United States) supplement with 10% (V/V) fetal bovine serum (FBS, Gibco, New York,

NY, USA), and 1% antibiotic-antimycotic solution penicillin/streptomycin (V/V) (Merck Millipore, MA, USA). Cultures were kept at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The cultured medium was refreshed every 3 days.

Compound preparation and protein-ligand interaction energy

NN01 or Mansonone G was extracted from the heartwood of *Mansonia gagei Drumm*, whereas other ether derivatives, NN02 and NN04, were semi-synthesized according to the previous study (75). SJ006 and SJ007 were synthesized from Lapachol which was extracted from *Usnea Lichen* (Old Man's Beard). All solid compounds were kept in a dark dry place at room temperature. The compounds were dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) to reach the final concentration of 10 mM and kept at 4°C for further experiments. Crystal structures of human G6PD (PDB ID: 7SNF) was obtained from Protein Data Bank (PDB). Three-dimension structure of–derivative compounds of 1,2-NQ and of G6PD inhibitors including DHEA and 6-AN were accessed the protein-ligand interaction energy. The interaction energy was generated using CDOCKER module implemented in Accelrys Discovery Studio 2.5 (Accelrys Inc.). G6-P, a substrate of G6PD, was a positive control.

Cytotoxic concentration (CC_{50}) and cells viability assay

Cells were seeded into 96-well plates at a density of 5 x 10^3 cells per well overnight. Different concentrations of derivative compounds of 1,2-NQ were treated

to test CC_{50}. After 48 hr of compound treatments, cells were incubated with 25 μl of 2 mg/mL MTT (Sigma-Aldrich, St. Louis, MO, USA) containing DMEM and incubated at 37°C for 2 hr. The MTT containing medium was removed, and the formazan dye was dissolved in 75 μl of DMSO. Cell viability was measured at 570 nm by the Synergy HT microplate reader (BioTek instruments Inc., USA). Each experiment was carried out in triplicates and the results were calculated as %cells viability of control.

To verify the inhibitory effect of derivative compounds of 1,2-NQ, D-(−)-ribose (R9629; Sigma-Aldrich) was dissolved in medium and filtrated to sterilize through a 0.22 μm filter membrane prior to use. After adding 5 mM D-(−)-ribose for 24 hr, the medium was removed prior to incubation with the derivative compound of 1,2-NQ for 48 hr.

G6PD activity assay, inhibitor test, and western blot analysis

Non-toxic concentrations of derivative compounds of 1,2-NQ were used to investigate the inhibition effect on G6PD. Cells were seeded into T-25 cm^3 flasks at a density of 8.75 x 10^4 cells per flask for 24 hr and then treated with non-toxic concentrations of each compound. After 48 hr of treatment, cells were trypsinized (Trypsin, HyClone, Logan, UT, USA) and pellets were washed with 1 X PBS and sonicated. Protein concentrations (mg/ml) were quantitated using the PierceTM BCA Protein Assay Kit (Thermo Fisher Scientific, MA, USA).

Ten µl of cell suspensions were mixed with 490 µl of G6PD reaction buffer as described previously (55). Finally, NADPH production was kinetically measured at 340 nm at 37°C on the Synergy HT microplate reader (BioTek instruments Inc., USA). G6PD activity was calculated with the NADPH standard curve in absorbance units per min per mg protein to demonstrate as an EC50 of compound. In order to determine K_m and V_{max} values during the identifying the type of inhibition, kinetic studies for G6PD activity measurement were performed under various concentrations of G6-P as previously described (55). A Lineweaver-Burk plot was conducted using the values of K_m and V_{max} in the presence of 6-AN, DHEA, and SJ006 to distinguish the type of inhibition.

Thirty micrograms of proteins were loaded separately into 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) and then transferred into the nitrocellulose membranes. After blocking for 1h with 5% non-fat milk in TBST (TBS-1% Tween20), membranes were incubated overnight with anti-G6PD (1:2,500) (Sigma-Aldrich, St. Louis, MO, USA) in TBST buffer at 4°C. Next, membranes were washed three times for 5 min with TBST buffer and probed with anti-Rabbit horseradish peroxidase conjugated secondary antibody for 2 h at room temperature. Lastly, membranes were washed thrice for 5 min each with TBST before detection of the interested protein using an enhanced chemiluminescence (ECL) system (Thermo Fisher Scientific, MA, USA) by using UVP Chemstudio Western Blot Imaging Systems by Analytik Jena (Analytik Jena™ UVP Chemstudio PLUS, Germany). The intensity of

protein bands was quantitated by VisionWorks software (Analytik Jena™ UVP Chemstudio PLUS, Germany) which ß-actin were used to normalize as an internal control.

Quantitative real-time PCR (qRT-PCR) analysis

Total RNA was isolated and quantitated using Trizol reagent (Thermo Fisher Scientific, MA, USA) and a Nanodrop 1000 spectrophotometer (Thermo Fisher Sciencetific, MA, USA), respectively. Real-time PCR analysis was performed to assess G6PD, Bax, and Bcl-2 mRNA expression. RNAs were converted into cDNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Sciencetific, MA, USA) according to the manufacturer's protocol. The expressions of mRNAs were measured using SYBR green mastermix (PowerUp™ SYBR® Green Master Mix (Thermo Fisher Sciencetific, MA, USA) in a StepOnePlus Real-Time PCR machine (Applied Biosystem, USA). Primers to amplify G6PD, Bax, and Bcl-2 cDNA were presented as follows: G6PD forward primer 5'-GTCAAGGTGTTGAAATGCATC-3' and reverse primer 5'-CATCCCACCTCTCATTCTCC-3', Bax forward primer 5'-AACATGGAGCTGCAGA GGAT-3' and reverse primer 5'-CAGCCCATGATGGTTCTGAT-3', Bcl_2 forward primer 5'-GGTGGGGTCATGTGTGTG-3' and reverse primer 5'- CGGTTCAGGTACTCAGTCATC-3', and ß-actin as a reference gene, forward primer 5'-ACTCTTCCAGCCTTCCTTC-3' and reverse primer 5'-ATCTCCTTCTGCATCCTGTC-3'. The fold changes of mRNA expression were calculated by the $2^{-\Delta\Delta Ct}$ method.

Reactive oxygen species (ROS) measurement by DCFH-DA assay

Cells were seeded in 96 black well plates at a density of 5 x 10^3 cells per well and allowed to attach overnight. Cells were then treated with the indicated concentrations of derivative compound of 1,2-NQ for 48 hr. The medium was replaced and incubated with 10 μM CM-H2DCFDA for 30-60 min at 37°C in the dark (Thermo Fisher Scientific, MA, USA). Intracellular ROS was detected by the oxidation effect of DCF-DA into the highly fluorescent compound DCF that can be measured at excitation/emission wavelengths of 488 nm/520 nm on the Synergy HT microplate reader (BioTek instruments Inc., USA).

Cell cycle analysis

Cells were seeded into 25 cm^3 flasks at a density of 8.75 x 10^5 cells overnight before treatment with the derivative compound of 1,2-NQ at the indicated concentrations for 48 hr. Cell cycle analysis was performed with a BD LSR II Flow Cytometer following the manufacturer's protocol (BD Biosciences, USA). After that, cell pellets were fixed with 70% ethanol (Merck Millipore, USA) for 30 min at 4°C. Then, 50 μg/ml propidium iodide (ImmunoTools, Germany) and 100 μg/ml RNase were used to stain the cells for 20 min at 4°C in the dark. Data was analyzed by BD FACSDiva™ software.

Statistical analysis

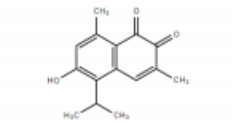
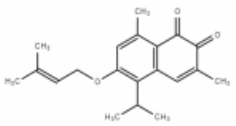
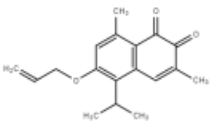
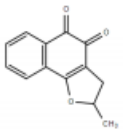
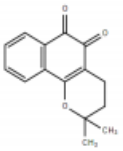
All statistical analyses were performed using SPSS Statistics version 22.0 (SPSS, Inc., Chicago, IL, USA). Non-linear regression analysis was used to examine effective concentration using the CC_{50} calculator program and GraphPad Prism 7.0 (GraphPad Software, Inc., USA). The statistical significance between each parameter was determined by one-way analysis of variance (ANOVA). Data were presented as mean ± standard error of the mean (SEM) and all statistical tests significant determined with *p*-values less than 0.05.

Results

Characteristics and cytotoxic concentrations (CC_{50}) of derivative compounds of 1,2-NQ

Characteristics of five 1,2-NQ derivative compounds, including NN01, NN02, NN04, SJ006, and SJ007 were presented in **Table 1.** Determination of the ability of derivative compounds of 1,2-NQ to bind to G6PD showed that NN01, NN02, NN04, SJ006, and SJ007 had better binding affinity to G6PD than 6-AN and were comparable to that of DHEA (**Figure 1**).

Table 1 Characteristics, including the International Union of Pure and Applied Chemistry name (IUPAC), structure, sources, and molecular weight (MW) of derivative compounds of 1,2-NQ.

Code	International Union of Pure and Applied Chemistry Name (IUPAC)	Structure	Source	MW (g/mol)
NN01	6-hydroxy-5-isopropyl-3,8-dimethyl-1,2-naphthalenedione (Mansonone G)		*Mansonia gagei Drumm*	244
NN02	5-isopropyl-3,8-dimethyl-6-[(3-methyl-2-buten-1-yl)oxy]-1,2-naphthalenedione		*Mansonia gagei Drumm*	312
NN04	6-(allyloxy)-5-isopropyl-3,8-dimethyl-1,2-naphthalenedione		*Mansonia gagei Drumm*	284
SJ006	2-methyl-2,3-dihydronaphtho [1,2-b]furan-4,5-dione		*Usnea Lichen* (Old Man's Beard)	214
SJ007	2,2-dimethyl-3,4-dihydro-2H-benzo[h]chromene-5,6-dione (β-lapachone)		*Usnea Lichen* (Old Man's Beard)	242

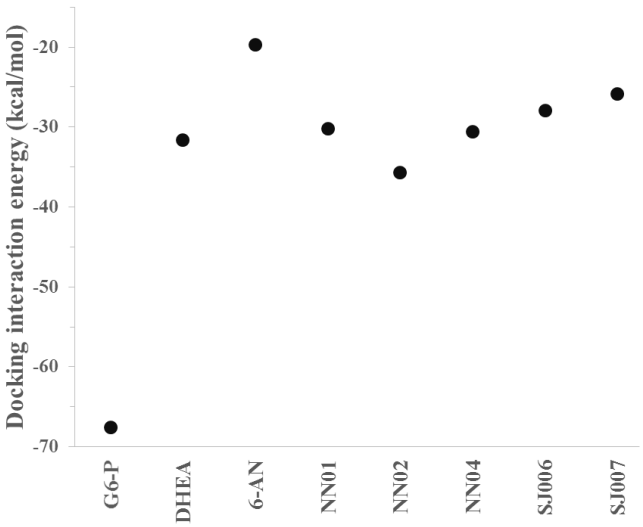


Figure 1 Molecular docking energy of five 1,2-NQ-derivative compounds (NN01, NN02, NN04, SJ006 and SJ007) compared to G6-P (substrate for G6PD) and known G6PD inhibitors (DHEA and 6-AN)

As CC_{50} values are shown in **Figure 2A-E** and **Table 2**, all five compounds exhibited a cytotoxic effect on A549 and H292 in a dose-dependent manner. H292 required a slightly higher concentration of compounds than A549 to reach CC_{50}. After 48 hr of treatment, NN02 was indicated as a high toxic compound in this study due to the highest cytotoxicity in A549 and H292 was shown, whereas NN04, SJ006, and SJ007 were in groups with less cytotoxicity in both cell lines compared to other compounds. In the H292 assay, the CC_{50} of SJ007 was predicted (**Figure 2E**), since the maximum dose of SJ007 (30 μM) could not reduce cell viability by 50% confluence. The appropriate concentrations, not more than CC_{50}, of each compound were then selected to examine the inhibition effect of G6PD activity.

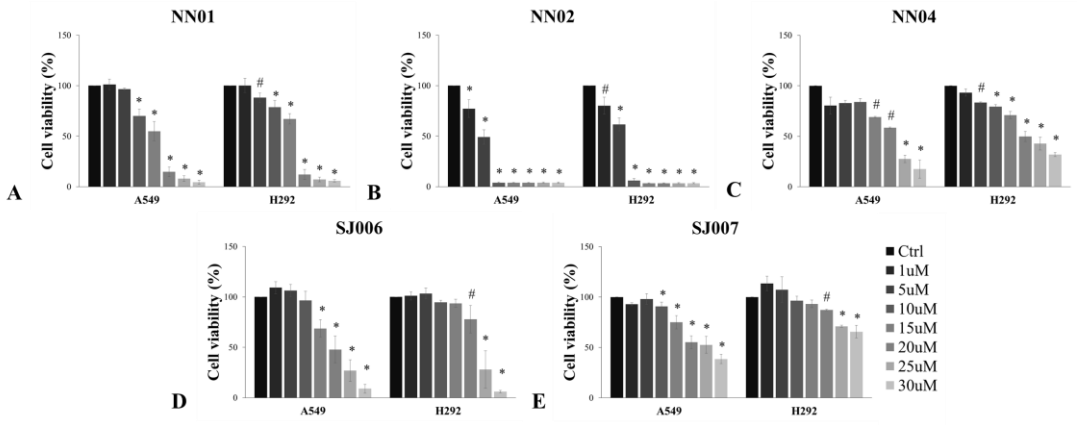


Figure 2 Cytotoxic concentration (CC_{50}) of derivative compounds of 1,2-NQ; (**A**) NN01, (**B**) NN02, (**C**) NN04, (**D**) SJ006, and (**E**) SJ007 in A549 and H292 cell lines. Data were represented as the means ± SD (n=3). Significant differences compared to control (Ctrl) were indicated as $^{\#}p<0.050$ and $^{*}p<0.001$.

Table 2 Cytotoxic concentration (CC_{50}) in five derivative compounds of 1,2-NQ

1,2-Naphthoquinone derivatives	Cytotoxic concentration (CC_{50}) (μM)	
	A549	H292
NN01	15.12 ± 1.49	15.43 ± 1.81
NN02	5.37 ± 0.38	6.27 ± 0.34
NN04	23.02 ± 2.09	23.56 ± 3.14
SJ006	21.65 ± 3.60	25.20 ± 4.47
SJ007	20.07 ± 4.62	32.86 ± 6.88*

* The CC_{50} was predicted, since the maximum dose (30 μM) could not reduce cell viability by 50% confluence.

Data are presented as the means ± SD (n = 3).

The inhibitory effects of 1,2-NQ derivative compounds on G6PD activity in NSCLC cell lines were presented in **Figure 3A-E**. After 48 hr treatment with the appropriate concentrations of each 1,2-NQ derivative compounds, only SJ006 reduced G6PD activity in a dose-dependent manner in both cell lines, at 10, 15 and 20 μM. NN01 and SJ007 also had the potency to inhibit G6PD activity but only in A549 cells. NN02 and NN04 had no inhibitory effect on G6PD activity in NSCLC cell lines and were more prone to induce G6PD activity in a dose-dependent manner. The levels of G6PD mRNA and protein expression were evaluated to affirm the direct inhibitory effect of SJ006 on G6PD activity without affecting G6PD expression. SJ006 had no effect on the expression of G6PD, both mRNA (**Figure 4A**) and protein (**Figure 4B**) in A549 and H292 cells.

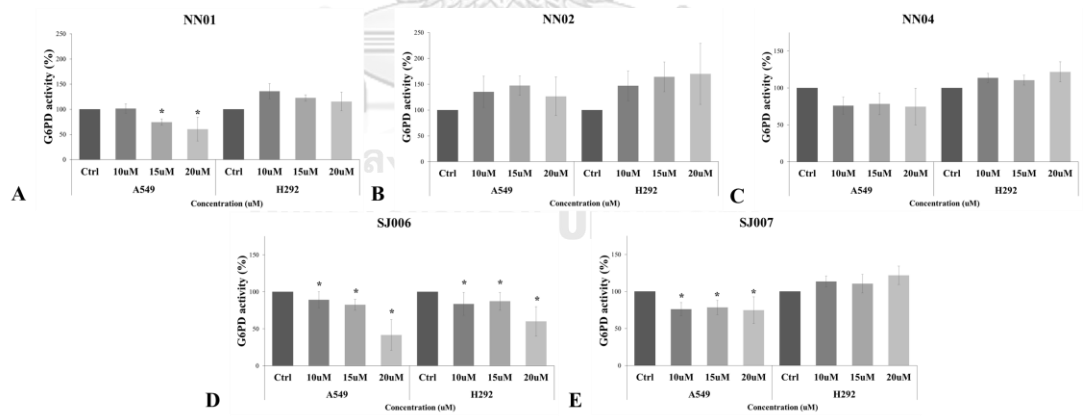


Figure 3 An inhibitory effect on G6PD activity of five derivative compounds of 1,2-NQ; (**A**) NN01, (**B**) NN02, (**C**) NN04, (**D**) SJ006, and (**E**) SJ007 in A549 and H292 cell lines. Data were represented as the means ± SD (n=3), and significant differences compared to control (Ctrl) were indicated as *p<0.05.

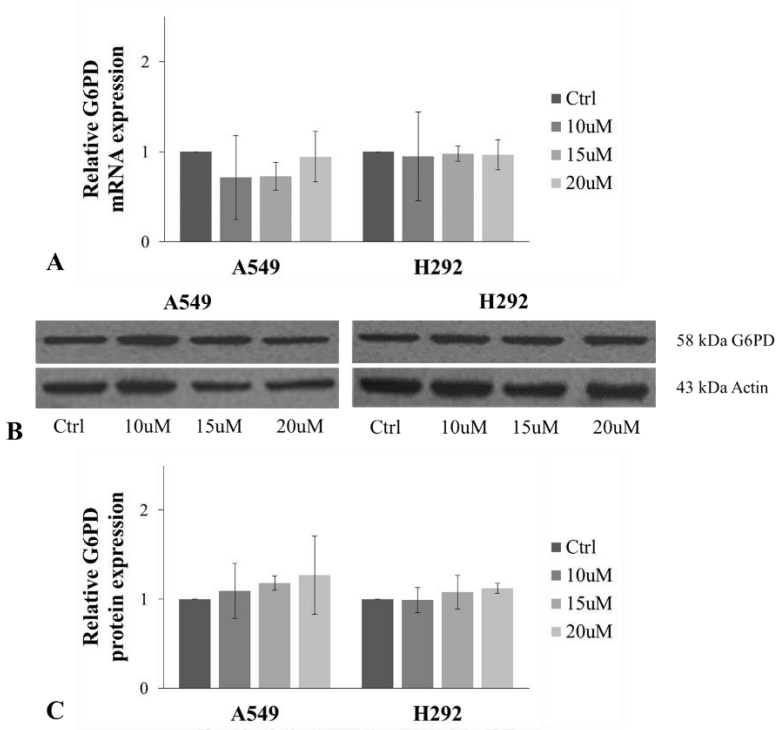


Figure 4 The level of G6PD (**A**) mRNA and (**B**) protein expression in A549 and H292 cell lines after treatment with SJ006. Data were represented as the means ± SD (n=3).

A kinetic study of the inhibition of the G6PD enzyme by the candidate 1,2-NQ derivative compounds, SJ006, was compared with that of known inhibitors (6-AN or DHEA). In the present of inhibitors (6-AN, DHEA and SJ006), the Michaelis-Menten equation and the enzyme-substrate-inhibitor relationship can be graphically analyzed using Lineweaver-Burk plots parallel with control (no inhibitor). Our findings found that 6-AN and DHEA exhibited competitive and noncompetitive inhibition of the G6PD enzyme, respectively (35, 76). As demonstrated in Lineweaver-Burk plot analysis and the elevation of K_m with unaffected V_{max} resulting in a competitive

inhibitor type of 6-AN (**Figure 5A** and **Table 2**). Moreover, the reduction of V_{max} without interfering K_m was observed and indicated as a noncompetitive inhibitor type of DHEA (**Figure 5B** and **Table 2**). Interestingly, the uncompetitive inhibitor type was demonstrated in SJ006 as the reduction in both K_m and V_{max} (**Figure 5C** and **Table 2**). Therefore, SJ006 was a derivative compound of 1,2-NQ that exhibited an uncompetitive inhibition of the G6PD enzyme with no effects on G6PD expression at both mRNA and protein levels.

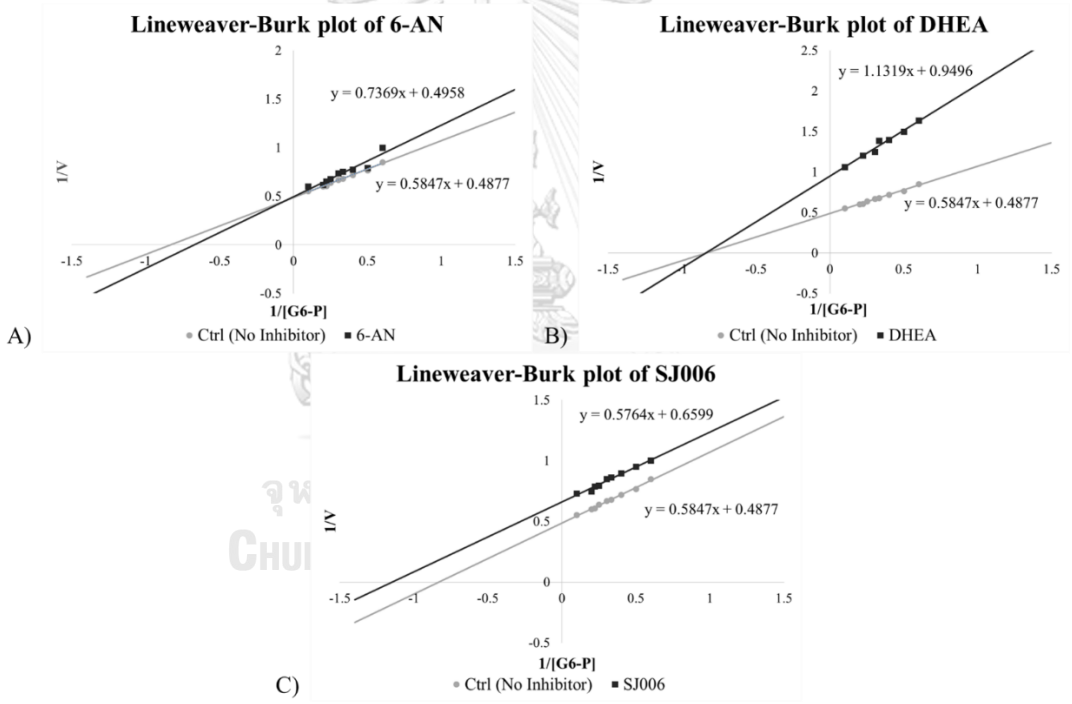


Figure 5 Lineweaver-Burk plot of G6PD inhibitors: (**A**) 6-AN, (**B**) DHEA, and (**C**) SJ006.

Table 3 K_m and V_{max} values for G6PD activity inhibition by addition of each inhibitor; 6-AN, DHEA and SJ006

Compound G6PD Inhibitor (Type of inhibitor)	Km (nmol)	Vmax (nmol/min)
Ctrl (No Inhibitor)	1.20	2.05
6-AN (Competitive)	1.49	2.02
DHEA (Noncompetitive)	1.19	1.05
SJ006 (Uncompetitive)	**0.87**	**1.5**

Effects of SJ006 on antiproliferation by inhibiting G6PD function.

Since SJ006 exhibited antiproliferation by inhibiting G6PD, an enzyme controlling redox homeostasis, the mechanisms by which SJ006 inhibited NSCLC proliferation through reactive oxygen species (ROS) induction, cell cycle arrest, activation of the apoptosis makers Bax/Bcl-2 mRNA expression, and rescue of SJ006-treated cell viability by D-(−)-ribose, the end product of PPP, were thus investigated. ROS production was increased in SJ006-treated A549 and H292 cell lines (**Figure 6A**). The levels of ROS were significantly increased in A549 cells at concentrations of SJ006 at 15 and 20 μM, (4.39 and 10.19 times relative to control, respectively), whereas in H292 cells, only a significant difference was observed at a high concentration of SJ006 at 20 μM (3.3 times relative to control).

Our results in cell cycle monitoring in A549 and H292 cells paralleled the increase in ROS production at high concentrations of SJ006 (**Figure 6B**). In A549 cells after treatment with SJ006 at concentrations of 15 and 20 μM, the G_0/G_1 phase was significantly decreased (9.49% and 11.52% relative to control, respectively), while the G_2/M phase was significantly increased (11.37% and 9.50%). After treatment with SJ006 at a concentration of 20 μM, the G_0/G_1 phase was significantly reduced in H292 cells (7.97% relative to control) where the G_2/M phase was significantly increased (7.22% relative to control).

The effect of SJ006 on cell apoptosis through the inhibition of G6PD was evaluated by the level of Bax/Bcl-2 mRNA ratio (**Figure 6C**). The significant increase Bax/Bcl-2 mRNA levels were clearly observed in SJ006-treated A549 cells at concentrations of 10, 15 and 20 μM (3.24, 2.96, 5.59 times relative to control, respectively) and H292 cells at concentrations of 20 μM (2.47 times relative to control).

D-(−)-ribose, an end product of PPP, was used to verify the inhibitory effect of SJ006 on cancer cell proliferation through inhibition of G6PD function. After A549 and H292 cells were treated with SJ006, the cell viability of D-(−)-ribose-pretreated cells tended to increase, starting at a low concentration of 15 μM but not reach significant (9.38% and 9.77%, respectively), which can be significantly observed at a

concentration of 20 μM (9.69% and 25.57%, respectively) compared to untreated D-(–)-ribose cells (**Figure 6D**).

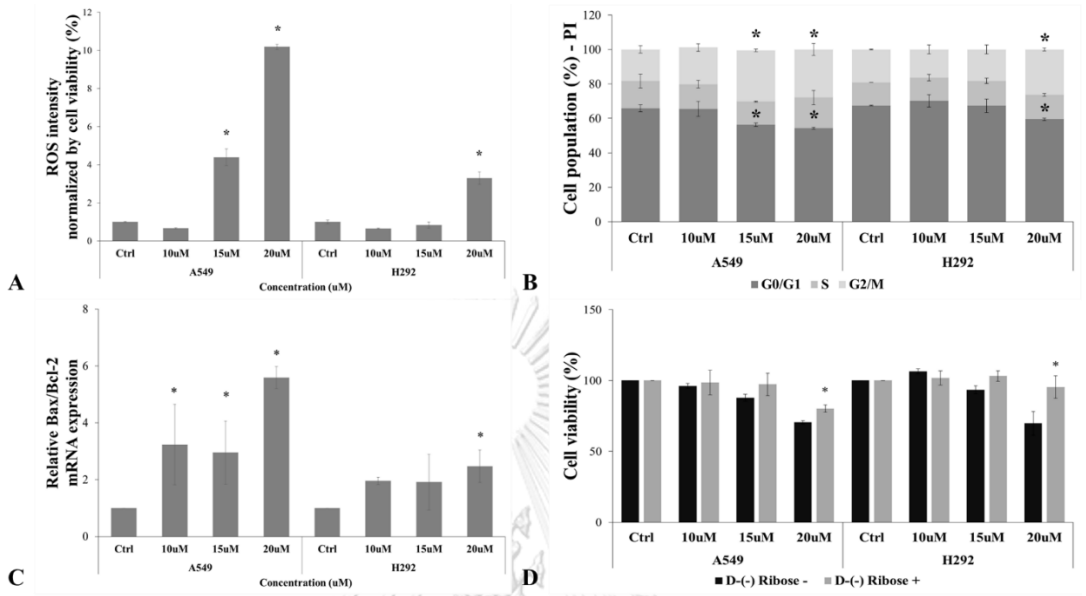


Figure 6 Effects of SJ006 on antiproliferation by inhibiting G6PD function. The mechanism by which SJ006 inhibited A549 and H292 cell proliferation through (**A**) ROS induction, (**B**) cell cycle arrest at G2/M phase, (**C**) activation of the apoptosis makers Bax/Bcl-2 mRNA expression, and (**D**) rescue of SJ006-treated cell viability by D-(–)-ribose, the end product of PPP. Data are represented as the means ± SD (n = 3), and significant differences are indicated as *p<0.05.

Discussion and conclusion

G6PD, in addition to its role in protecting red blood cells from oxidative damage, has also been implicated in cancer biology. Several studies suggest that G6PD may play a dual role in cancer development and progression (2, 77, 78). On

one hand, G6PD can promote tumor growth by providing cancer cells with an increased supply of NADPH, which is essential for cell proliferation and survival. Elevated G6PD activity has been observed in various types of cancer, including lung cancer, and it is believed to contribute to the increased antioxidant capacity and resistance to oxidative stress exhibited by cancer cells (10). G6PD has been recognized as one such emerging hallmark due to its involvement in various aspects of cancer biology including redox balance, proliferative advantage, metabolic reprogramming, and chemoresistance (2). It also highlights G6PD as a potential therapeutic target for developing novel anti-cancer strategies aimed at disrupting metabolic vulnerabilities and overcoming treatment resistance. Here, we characterized derivative compounds of 1,2-NQ as novel G6PD inhibitors with anticancer properties in NSCLC cells.

Our studies demonstrated that five derivative compounds of 1,2-NQ had a higher binding affinity for G6PD than 6-AN. 6-AN was first reported as a generic competitive G6PD inhibitor (33) and induces oxidative stress in cancer cells (10). Our findings are consistent with Kohler E., et al. and Aurora AB, et al. that it is able to bind to NADP+ (35) and 6-PGD (36) instead of G6PD. The lack of specificity of 6-AN may affect other cellular processes and targets. This can lead to unintended consequences and side effects that may limit its therapeutic potential. Moreover, compounds that are derivatives of 1,2-NQ showed a binding affinity for G6PD comparable to that of DHEA. DHEA, a known non-competitive G6PD inhibitor, is a

precursor hormone that can be converted into other hormones, including estrogen and testosterone (79). This multifunctional hormone can have various effects on the body, including potential promotion of hormone-sensitive cancers (30) and off-target effects including mitochondrial alteration (31, 32). It is crucial to consider the potential risks when using DHEA as a cancer treatment. Five compounds that are derivatives of 1,2-NQ were therefore investigated for their properties in the inhibition of G6PD function and anticancer activity in NSCLC cells.

This study revealed for the first time that SJ006, 2-methyl-2,3-dihydronaphtho [1,2-b]furan-4,5-dione, is a novel G6PD inhibitor for NSCLC cells, at least for adenocarcinomic human alveolar basal epithelial cells (A549) and human lung mucoepidermoid carcinoma cells (NCI-H292). SJ006 is a 1,2-naphthoquinone-containing compound extracted from *Lichen*. It decreased both K_m and V_{max} of the G6PD enzyme, indicating that it is an uncompetitive inhibitor for the G6PD enzyme. SJ006 has a direct effect on reducing G6PD activity without affecting the level of G6PD mRNA and protein expression. NN01 and SJ007 had the potential to inhibit G6PD activity, but the effect was observed only in A549 cells. This may be because the basal expression level of G6PD enzyme in NCI-H292 cells was higher than that in A549 cells, affecting the optimum concentration of NN01 and SJ007 to inhibit the G6PD enzyme in NCI-H292 cells, which should be higher. However, testing with concentrations of these compounds above CC_{50} decreases cell viability. This resulted

in insufficient cell palletization for the enzyme kinetic assay. Unfortunately, NN02 and NN04 seemed to have no inhibitory effects.

Previous studies suggested the role of derivative compounds of 1,2-NQ against cancer cell proliferation (38, 39, 41, 42). Safwat G., et al. indicated that SJ006 have anticancer activity in leukemic cell lines; HL-60 (80). In this study, all five derivative compounds of 1,2-NQ showed anti-proliferation in NSCLC cell lines in a dose-dependent manner. In this study, H292 (*KRAS* wild-type cells) exhibited higher levels of CC_{50} than A549 (*KRAS* mutant cells), which may imply that KRAS mutant cells are more sensitive to derivative compounds of 1,2-NQ than the wild-type cells. The previous study classified a group of NQ derivatives as active and non-active compounds by state CC_{50} (less than 20 μM) and considered NQ an active compound in the colorectal adenocarcinoma cell line (HT-29) (81). Therefore, all derivative compounds of 1,2-NQ used in this study were marked as active NQ compounds in lung cancer cell lines.

G6PD is an enzyme generating NADPH, a coenzyme that reduces cellular ROS (10, 82, 83). As expected, SJ006 increased cellular ROS in both A549 and H292 cells, inducing cell cycle arrest at G_0/G_1 and G_2/M phases in both A549 and H292 NSCLC cell lines. Elevation of ROS was considered a mechanism that induced cell cycle arrest and cellular damage in cancer cells (84, 85). G_0/G_1 and G_2/M arrest have been demonstrated here for the first time as an effect of a derivative compound of 1,2-

NQ. Previous study suggested that the reduction of the G0/G1 phase but increase in the G2/M phase might be a repair state of the cell cycle (86). Moreover, the accumulation of subG_1 has been observed in this study supporting a role of 1,2-NQ, SJ006, on cell cycle arrest and apoptosis (87). Cellular apoptosis markers Bax/Bcl-2 were then evaluated to verify more on the cytotoxic of 1,2-NQ derivatives on NSCLC cells. SJ006 induced cellular apoptosis markers Bax/Bcl-2 in both A549 and H292 cells. Previous study by Zugic A., et al. and Disoma C EM., et al. indicated that phenolic compound from *Lichen* induces ROS production (88) and apoptosis (89). de Almeida PDO., et al. demonstrated that NQ induces ROS-mediated apoptosis through the activation of the JNK and p38 signaling pathways in breast cancer cell lines, MCF-7 (90). Interestingly, G2/M cell cycle arrest, and apoptosis were suggested as effects mediated through ROS accumulation (91). This phenomenon might be suggested as one of the anticancer activities of the 1,2-NQ compound (39, 41).

Our study investigated whether D-(−)-ribose reversed the anti-proliferative effects of SJ006 through the inhibition of G6PD in both A549 and H292 cells. D-(−)-ribose has been used as a supplement to investigate the proliferative effect in many studies (66, 92, 93). Different concentrations of D-(−)-ribose have been used in various cancer cell types (66, 67). Although there was no study between ribose and G6PD, the evidence in this study suggested that SJ006 affects the inhibition of G6PD activity. The mechanism by which cells generate D-(−)-ribose through PPP is necessary for the generation of ATP has been reported and suggested that impaired cellular metabolic

processes may be remedied by D-(−)-ribose supplementation (94). Therefore, the mechanism of D-(−)-ribose rescuing cancer cell proliferation in NSCLC cell lines in this study might indicate the impact of G6PD suppression on metabolic reprogramming through the inhibition by SJ006.

Our present study demonstrates the anticancer activity of SJ006, 2-methyl-2,3-dihydronaphtho[1,2-b]furan-4,5-dione, through its inhibitory effects directly on G6PD activity in both A549 and H292 NSCLC cells. Moreover, our study suggested that A549 (KRAS mutant) was more susceptible to the anticancer effects of SJ006 than H292 (KRAS wild type). Further investigations should validate the role of other 1,2-NQ compounds (NN01 and SJ007) in the inhibition of G6PD activity and verify the mechanism of SJ006 in *KRAS* mutation. Moreover, the impact of the anticancer effects of 1,2-NQ compounds and their side effects should be further investigated in G6PD overexpression cancer cells containing the KRAS mutation and animal models. Moreover, combination treatment of SJ006 with chemotherapy drugs is interesting to be investigated in G6PD overexpression cancer cells. This study can be concluded that SJ006 could be a novel uncompetitive G6PD inhibitor with anti-cancer activities in NSCLC cells.

Abbreviations

G6PD: Glucose 6-phosphate dehydrogenase

NSCLC: Non-small cell lung cancer

SCLC: Small cell lung cancer

NADPH: Nicotinamide adenine dinucleotide

EMR: Energy metabolic reprograming

PPP: Pentose phosphate pathway

ROS: Reactive oxygen species

Ethics approval and consent to participate

This study was approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University (IRB No.030/65). The protocol of this study was performed according to the Declaration of Helsinki for the participation of human individuals.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

CLC designed the research study. MC performed cell culture research, analyzed, and interpreted the data and drafted the manuscript. CLC and PC revised the manuscript. All authors read and approved the final manuscript.

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Section 3

3.1 Conclusions

The overexpression of G6PD was restricted to NSCLC tissues compared to SCLC tissues. The expression level of G6PD in NSCLC cells was positively associated with cell proliferation. The study indicated that disruption of G6PD function in NSCLC cells using DHEA and G6PD siRNA decreased cell proliferation and migration and increased apoptosis. D-(-) ribose, the end product of PPP, reversed the anti-proliferation in DHEA and G6PD siRNA-treated NSCLC cells. These findings support the idea that G6PD is a potential therapeutic target for developing novel anticancer properties in NSCLC. SJ006 or 2-methyl-2,3-dihydronaphtho [1,2-b]furan-4,5-dione 1,2-naphthoquinone, a novel uncompetitive G6PD inhibitor that reduces K_m and V_{max}, was identified. SJ006 had anticancer properties by reducing cell proliferation through the inhibition of G6PD activity was demonstrated. Moreover, the increase of ROS levels has been observed, promoting apoptosis and activating cell cycle arrest at the G2/M phase in both *KRAS* mutant and *KRAS* wild-type NSCLC cell lines.

3.2 Limitations

Determining the effective concentration (EC_{50}) of the derivative compounds of 1,2-NQ in inhibiting G6PD activity was a limitation of this study. The G6PD protein concentration that was retrieved from the treated cells was inadequate for the study of EC_{50} due to the cytotoxicity of compounds. Therefore, the cytotoxicity

concentration (CC_{50}) assay, together with monitoring G6PD inhibition, was used instead.

3.3 Suggestions for future research

SJ006 is a novel uncompetitive G6PD inhibitor, containing anticancer properties in NSCLC cells. Our study suggested that A549 (*KRAS* mutant NSCLC cells) was more susceptible to the anticancer effects of SJ006 than H292 (KRAS wild-type NSCLC cells). The impact of the anticancer effects of SJ006 and its side effects should be further investigated in G6PD overexpression cancer cells containing the *KRAS* mutation and animal models. Moreover, combination treatment of SJ006 with chemotherapy drugs is interesting to be investigated in G6PD overexpression cancer cells.

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