PROTECTIVE EFFECT OF PLAUNOTOL IN DOXORUBICIN-INDUCED APOPTOSIS IN HUMAN RENAL HK-2 CELLS THROUGH ANTIOXIDANT MECHANISMS

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A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Pharmaceutical Technology
Department of Pharmaceutics and Industrial Pharmacy
Faculty of Pharmaceutical Sciences
Chulalongkorn University
Academic Year 2012

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เป็นแฟ้มข้อมูลของนิสิตเจ้าของวิทยานิพนธ์ที่ส่งผ่านทางบัณฑิตวิทยาลัย
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นายฉัตรชัย เชาว์ธรรม

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาเทคโนโลยีเภสัชกรรม ภาควิชาวิทยาการเภสัชกรรมและเภสัชอุตสาหกรรม
คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2555
ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

Thesis Title	PROTECTIVE EFFECT OF PLAUNOTOL IN DOXORUBICIN-INDUCED APOPTOSIS IN HUMAN RENAL HK-2 CELLS THROUGH ANTIOXIDANT MECHANISMS
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ฉัตรชัย เชาว์ธรรม : ผลของเพลาโนทอลต่อการป้องกันอะพ็อพโทซิสที่เหนี่ยวนำโดยด็อกโซรูบิซินในเซลล์ไตชนิดเอชเค- 2 ของมนุษย์โดยกลไกต้านออกซิเดชัน (PROTECTIVE EFFECT OF PLAUNOTOL IN DOXORUBICIN-INDUCED APOPTOSIS IN HUMAN RENAL HK-2 CELLS THROUGH ANTIOXIDANT MECHNISMS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : รศ. ดร.วันชัย ดีเอกนามกูล , อ.ที่ปรึกษาวิทยานิพนธ์ร่วม : ผศ. ภก. ดร.ปิติ จันทร์วรโชติ, 102 หน้า.

ในการค้นหาสารที่มีความปลอดภัยและประสิทธิภาพเพื่อใช้ในเคมีบำบัดและสามารถยับยั้งความเป็นพิษของด็อกโซรูบิซินต่อเซ ลล์ไตได้นั้น จึ งมีการศึกษาความเป็นไปได้ที่จะนำเพลาโนทอลซึ่งเป็นสารสำคัญสกัดจากต้นเปล้าน้อยมาใช้ ในการทดลองครั้งนี้ผลการศึกษาพบว่าเพลาโนทอล สามารถป้องกันการตายของเซลล์ท่อไตส่วนต้นของมนุษย์ที่เกิดขึ้นจากด็อกโซรูบินซินได้ ด็อกโซรูบิซินลดการมีชีวิตของเซลล์ไตได้อย่างมีนัยสำคัญ ซึ่งส่วนใหญ่เนื่องมาจากการตาย แบบอะพ็อพโทซิส การเติมเพลาโนทอลในการเลี้ยงเซลล์เป็นเวลาอย่างน้อย 9 ชั่วโมงก่อน ทดสอบด้วยด็อกโซรูบิซิน สามารถทำให้เซลล์ อยู่รอดจากความเป็นพิษของด็อกโซรูบิซิน โดยอาศัยกลไกของเพลาโนทอลที่เพิ่มโปรตีนต้านอะพ็อพโทซิสชนิดเอ็มซีแอล-1 ผลการทดลองแสดงอย่างชัดเจนว่าการลดลงของระดับเอ็มซีแอล -1 หลังจากการ ได้รับด็อกโซรูบิซินนั้นมีความสัมพันธ์อย่างใกล้ชิดกับความ เป็นพิษของยา และการเพิ่มขึ้นของเอ็มซีแอล -1โดยการเลี้ยงเซลล์ไต ด้วย เพลาโนทอลสามารถ ป้องกันไม่ให้เซลล์ตายจากการเหนี่ยวนำของด็อกโซรูบิซินได้ นอกจากนี้ยังพบว่าการลดลงของเอ็มซีแอล -1 มีความ สัมพันธ์ อย่างใกล้ชิดกับกา รเกิด ของอนุพันธ์ออกซิเจนที่ว่องไว สารต้ านออกซิเดชันและเพลาโนทอล ป้องกันการลดลงของระดับเอ็มซีแอล -1 ที่เกิดจากการเหนี่ยวนำของอนุพัน ธ์ออกซิเจนที่ว่องไวได้ การเ ลี้ยงเซลล์ ด้วยเพลาโนทอลนาน 3 ชั่วโมง สามารถออกฤทธิ์ต้านออกซิเดชันในเซลล์ไตของมนุษย์ การลดของอนุ พันธ์ออกซิเจนที่ว่องไวในเซลล์ไตเป็นผลมาจากการเพิ่มขึ้นของกลูตาไธโอน ซึ่งถูกเหนี่ยวนำโดยการเ ลี้ยงเซลล์ ด้วยเพลาโนทอล ผ ลการทดลองเหล่านี้ชี้ให้เห็นว่า การป้องกันเซลล์ไตของเพลาโนทอล เป็นผลจากฤทธิ์ต้าน ออกซิเดชันซึ่ง ทำให้โปรตีนต้านอะพ็อพโทซิสชนิดเอ็มซีแอล-1 เพิ่มขึ้น

ภาควิชา วิทยาการเภสัชกรรมและเภสัชอุตสาหกรรม ลายมือชื่อนิสิต .....

สาขาวิชา เทคโนโลยีเภสัชกรรม ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

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5077104833 : MAJOR PHARMACEUTICAL TECHNOLOGY
KEYWORDS : PLAUNOTOL / DOXORUBICIN / MCL-1 / NEPHROTOXICITY / ANTIOXIDANT / REACTIVE OXYGEN SPECIES

CHATCHAI CHAOTHAM: PROTECTIVE EFFECT OF PLAUNOTOL IN DOXORUBICIN-INDUCED APOPTOSIS IN HUMAN RENAL HK-2 CELLS THROUGH ANTIOXIDANT MECHNISMS. THESIS ADVISOR : ASSOC. PROF. WANCHAI DE-EKNAMKUL, Ph.D., THESIS CO-ADVISOR : ASSIST. PROF. PITHI CHANVORACHOTE, Ph.D., 102 pp.

In searching for the safe and effective compounds to be used as chemoprotective agents to cease toxicity of doxorubicin on renal cells, plaunotol which is a major constituent in *Croton stellatopilosus* Ohba extract was studied for its possible effect. The results showed that plaunotol exhibited the protection against doxorubicin-induced cell death in human proximal tubule cells. Treatment of the renal cells with doxorubicin resulted in the significant decrease of cell viability, mainly due to the cause of apoptosis. Pretreatment of the cells with plaunotol for at least 9 h prior doxorubicin exposure rendered cell survival against the toxicity of doxorubicin. In terms of mechanism, plaunotol was shown to up-regulate the level of anti-apoptotic Mcl-1. It was clearly showed that the decrease of Mcl-1 level after doxorubicin treatment was closely associated with the toxic action of the drug, and the pretreatment of plaunotol caused the increase of Mcl-1 was able to prevent the doxorubicin-induced cell death. Furthermore, it was found that the lowering of Mcl-1 was closely related to the presence of reactive oxygen species (ROS). Pretreatment of an antioxidant and plaunotol appeared to retrieve the Mcl-1 level from ROS-induced diminution. Antioxidant activity of plaunotol in human renal cells was clearly observed after the incubation with plaunotol for 3 h. The reduction of ROS in the cells appeared to come from the increase of cellular glutathione which was induced by plaunotol treatment. These results suggest that the nephroprotective effect of plaunotol is a result from its antioxidant activity which in turn preserves the level of anti-apoptotic Mcl-1 protein.

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Field of Study : Pharmaceutical Technology Advisor's Signature .....
Academic Year : 2012 Co-advisor's Signature .....

ACKNOWLEDGEMENTS

This dissertation would have not been completed without the great supervision and support from my advisors, committee members, colleagues, friend and family.

My deep gratefulness is first expressed to my advisor, Associate Professor Dr. Wanchai De-Eknamkul who kindly gave me a precious opportunity to pursue the Doctoral Degree and a memorably academic experience on the international stage. I would like to warmly thank to Assistant Professor Dr. Pithi Chanvorachote, my thesis co-advisor, who devoted himself for passing on the laboratory skills and conceptual thinking to me. Not only the research framework but also the spirit of giving and sharing was the best thing that I learned from him. My appreciation is stated to the thesis committee for their benefit comments that helped to improve and construct my dissertation.

I would like to express special thanks to my classmate and the staffs of Pharmaceutical Technology (International) Program for their good cooperation and assistance.

This dissertation is dedicated to my parents. The seeking of my Doctoral Degree could not be accomplished without their truly support and understanding in any regards.

Finally, my gratefulness is expressed to The Royal Golden Jubilee Ph. D. Program for grant support (5.Q.CU/50/C.1).

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LIST OF ABBREVIATIONS

~	approximately
°C	degree Celcius (centrigrade)
<	less than
%	percentage
μ	micro (10^{-6})
AAPH	2,2′-Azobis(2-methylpropionamidine) dihydrochloride
Bcl-2	B-cell lymphoma 2
COX2	cyclooxygenase 2
Cu/Zn-SOD	copper/zinc superoxide dismutase
Cu^{+}	cupric ion
Cu^{2+}	cuprous ion
DCF	dichlorofluorescein
DHE	dihydroethidium
DMTU	dimethylthiourea
DMNQ	2,3-dimethoxy-1,4-naphthoquinone
Dox	doxorubicin
DPPH	2,2-diphenyl-1-picrylhydrazyl
et al.	et alii, and other
Fe^{2+}	ferrous ion
Fe^{3+}	ferric ion
g	gram (s)
GSH	reduced glutathione

GPx	glutathione peroxidase
h	hour (s)
H_2O_2	hydrogen peroxide
H_2DCF-DA	dihydrodichlorofluorescein diacetate
HPF	hydroxyphenyl fluorescein
k	kilo (10^3)
l	litre (s)
m	milli (10^{-3})
M	molar (s), micromole (s) per litre
Mcl-1	myeloid cell leukemia-1
min	minute (s)
MnSOD	manganese superoxide dismutase
MnTBAP	Manganese (III) Tetrakis (4-Benzoic Acid) Porphyrin chloride
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAC	N-acetylcysteine
nM	nanometer
O_2	oxygen molecule
$O_2^{\bullet-}$	superoxide anion
$OH^{\cdot}$	hydroxyl radical
ORAC	oxygen radical absorbance capacity assay
PGE2	prostaglandin E2
PI	propidium iodide
Plau	plaunotol
ROS	reactive oxygen species

S.D.	standard deviation
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SOD	superoxide dismutase
TBST	tris-buffered saline with tween

CHAPTER I

INTRODUCTION

Renal injury has been notified as an incurrent disease in various pathobiologies including diabetes, hypertension, and chemotherapeutic treatment (Bergamini and Seghieri, 2006; Ichikawa, Kiyama, and Yoshioka, 1994; Walker, 1993). Drug related-nephrotoxicity can lead to the impairment of renal function and withdrawal of the efficient therapy (Yao et al., 2007). According to excretory organ and high exposure to toxic substance, deterioration of glomerular and tubular segment is mostly found in renal failure (Bonventre, 2010; Naughton, 2008).

Doxorubicin, an anthracyclin antibiotic, has been continuously acknowledged for its effectiveness in treatment of various human carcinomas (Lown, 1993). However, in certain cases, doxorubicin causes the unsought effects found as a serious degeneration of tissue component of heart and kidney (Mansour, El-Kashef, and Al-Shabanah, 1999; Saad, Najjar, and Al-Rikabi, 2001). Doxorubicin-induced nephrotoxicity is a dose-limiting toxicity with presence of dramatic increase of serum creatinin and atrophy of tubular renal cells (Burke et al., 1977; Kaur et al., 2012). Apoptotic cell death in renal injury is illustrated after administration of doxorubicin in both *in vitro* and *in vivo* model (Cheng et al., 2006; Ueda and Shah, 2000; Zhang et al., 1996). While the underlying mechanisms remain unclear, many substances have been proposed for the prevention on renal toxicity from doxorubicin (Boonsanit, Kanchanapangka, and Buranakarl, 2006; Boutabet et al., 2011; Chen et al., 2006).

Apoptosis is a programed cell death that is tightly regulated by pro-apoptotic and anti-apoptotic Bcl-2 family proteins. Overexpression of myeloid cell leukaemia-1 (Mcl-1), a pro-survival Bcl-2 protein, sufficiently protects toxicity from doxorubicin (Bolesta et al., 2012; Katoh et al., 1998; Lestini et al., 2009; Wei et al., 2001; Wirth et al., 2005; Yang et al., 2012). Mcl-1 is a short half-life protein which inhibits the pore formation on mitochondrial membrane and releasing of cytochrome *c* through binding and inactivating pro-apoptotic proteins including Bak, tBid, Bim and PUMA (Akgul, 2009; Johnson and Packham, 2005; Thomas, Lam, and Edwards, 2010). Rapid degradation of Mcl-1 can be provoked by reactive oxygen species (ROS) as indicated with the depletion of Mcl-1 in the oxidative stress condition (Blatt et al., 2009; Cheng et al., 2010; Um and Kwon, 2010). Meanwhile, immunochemistry reveals the up-regulation of Mcl-1 level in patients' tissue that highly expresses cellular antioxidant (Elbordiny et al., 2007). These evident imply that cellular oxidative stress has a crucial role in manipulation of Mcl-1 level. The expression of Mcl-1 level is distinct in different organs. Unfortunately, human renal tissue expresses low to moderate level of Mcl-1 especially in proximal tubule cells (Krajewski et al., 1995).

Due to the diverse pharmacological activities and human safety profile, plant terpenoid extracts have come to attention as a good source of natural medication (Paduch et al., 2007). Among these extracts, plaunotol, an acyclic diterpenoid extracted from leave of *Croton stellatopilosus* Ohba, is approved to be prescribed in human for anti-gastric ulcer treatment (Kaneko et al., 1995; Paduch et al., 2007; Wada et al., 1997). Cytoprotective mechanism of plaunotol involves with the increase of cyclooxygenase 2 (COX2) via NF-κB activation. The increase of COX2 stimulates prostaglandin E2 (PGE2) production which in turn controls gastric acid and mucosal

secretion (Fu et al., 2005). Not only this compound is considerably safe and has been used in the treatment of gastric ulcer for long time, but also plaunotol is shown to processes antioxidant activity in human blood cells (Okabe et al., 1995). Nevertheless the antioxidant and defensive capacity of plaunotol oppose to renal toxicity has not been studied. Additional with the distribution into renal tissue, the development of planotol as a renoprotective medicine is prosperously manifested.

In the literature, chemotherapeutic-induced nephrotoxicity has been reported to be account for the withdrawal of effective medication and the risk of mortality in cancer patients (Naughton, 2008; Pannu and Nadim, 2008; Perazella, 2009; Yao et al., 2007). Recently, various substances have been investigated for the protective effect against doxorubicin-induced nephrotoxicity but most of them still have not been clarified for the safety in human (Boonsanit et al., 2006; Boutabet et al., 2011; Chen et al., 2006). The use of plaunotol for this study has a number of advantages. These include human safety profile, high accumulation and distribution in renal tissue and antioxidant potential, these become the rational that plaunotol might prevent doxorubicin-induced nephrotoxicity. The objectives of this research aim to 1) evaluate the protective effect of plaunotol against doxorubicin-induced toxicity in human renal cells, and 2) investigate the underlying mechanisms of protective effect of plaunotol.

This study would bring to comprehensive knowledge of doxorubicin-induced nephrotoxicity, associate with preventive activity and underlying mechanisms of plaunotol against renal damage. The findings would support the development of this safe natural compound for novel application as a renoprotective drug.

CHAPTER II

LITERATURE REVIEW

Doxorubicin as antineoplastic drug

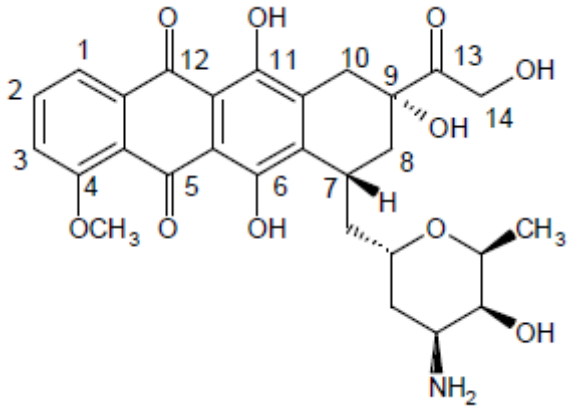


Figure 1. Chemical structure of doxorubicin (Kaur et al., 2012).

Among the most effective antineoplastic drugs, doxorubicin (Figure 1) has been recognized for its highly inhibitory effect against various cancer cells such as breast, lung and ovary cancer as well as leukemia's and Hodgkin's lymphoma (Kaur et al., 2012). Doxorubicin is categorized as an anthracycline antibiotic which possesses in antitumor activity. The production and isolation of doxorubicin has been preceded through the metabolism of *Streptomyces peucetius* bacteria (Aubel-Sadron and Londos-Gagliardi, 1984). Doxorubicin composes a red chromophore of anthraquinone structure and daunosamine sugar (Figure 1). These anthraquinone and daunosamine ring play a vital role in anticancer activity of doxorubicin (Aubel-

Sadron and Londos-Gagliardi, 1984; Lown, 1993). Similar to other highly potent chemotherapeutic drugs, the usefulness of doxorubicin is terminated by its dose-related toxicity especially on cardiac and renal tissue (Burke et al., 1977; Kaur et al., 2012; Saltiel and McGuire, 1983). The attempt to find a natural substance that prevents doxorubicin-induced toxicity has been reported from various studies (Boonsanit et al., 2006; Boutabet et al., 2011; Cheng et al., 2006; Granados-Principal et al., 2010). Although these natural extracts demonstrate the protective effect against doxorubicin-induced toxicity, the inquiry of safety profile in human and the obstruction on anticancer property of doxorubicin still need more clarification.

1. Doxorubicin mechanism of action

Although the underlying mechanism of doxorubicin-induced cytotoxicity still being discussed, there are various modes of action including DNA intercalation, topoisomerase II poison, generation of reactive oxygen species (ROS) and the influence of a survival protein have been of interests (Cutts et al., 2005; Gewirtz, 1999; Granados-Principal et al., 2010; Swift et al., 2006).

1.1 DNA intercalation

According to the chemical structure of doxorubicin (Figure 1), DNA intercalation has been proposed as one of anticancer mechanisms of this compound (Aubel-Sadron and Londos-Gagliardi, 1984; Lown, 1993). *In vitro* studies have demonstrated the interaction between doxorubicin and double strand DNA in cancer cells (Cutts et al., 2003; Skladanowski and Konopa, 1994; Taatjes, Fenick, and Koch,

1999). Doxorubicin can rapidly intercalate between double strands of nuclease DNA (Figure 2) resulting in the inhibition of protein synthesis and cell cycle arrest sequential with anti-proliferation in tumor cells and apoptotic programed cell death (Cutts et al., 1996; Swift et al., 2006). The interstrand DNA crosslink of doxorubicin starts with the activation at 3´ amino of daunosamine sugar by cellular formaldehyde then the covalent bond between daunosamine and guanine amino acid is generated. Meanwhile, two hydrogen bonds between anthraqunione ring and guanine base are also generated at another DNA strand. Although doxorubicin can interact with adenine and guanine base, the high affinity between doxorubicin and DNA strand is found in cytosine-guanine rather than adenine-thymidine base pair (Aubel-Sadron and Londos-Gagliardi, 1984; Lee et al., 2004; Zunino et al., 1980).

Since the pre-activation of doxorubicin is a crucial step in DNA-intercalation, the increase of cellular formaldehyde in tumor cells enhances antineoplastic specificity and efficacy of doxorubicin (Burke and Koch, 2001; Cutts et al., 2005; Fenick, Taatjes, and Koch, 1997; Taatjes et al., 1999).

1.2 Topoisomerase IIα poison

Topoisomerase IIα is an essential enzyme involving in DNA integrity, DNA replication, cell proliferation and cell survival. The main function of topoisomerase IIα is uncoiling the overwind double helix DNA and separation of daughter DNA which usually take place during the DNA replicating process. The unwinding activity of topoisomerase IIα depends on the transient breaking and religating at phosphate backbone of DNA strand (Fortune and Osheroff, 2000). Since topoisomerase IIα is necessary for cell division, all proliferative cells, especially cancer cells, highly

express topoisomerase IIα enzyme. There are many chemotherapeutic drugs that have been designed to impair this catalytic function of topoisomerase IIα (Burden and Osheroff, 1998). Doxorubicin has been introduced as a topoisomerase poisoning compound due to the over stimulation on topoisomerase IIα activity. DNA intercalation of doxorubicin not only inhibits DNA transcription but also induces topoisomerase IIα binding to the DNA adduct and create the intermediate complex of doxorubicin-DNA-topoisomerase IIα (Burden and Osheroff, 1998; Swift et al., 2006). This intermediate complex impairs topoisomerase IIα function though the inhibition of religation process of broken DNA strand. The accumulation of damage DNA consequently results in apoptosis induction (Gewirtz, 1999; Kik et al., 2009; Ramachandran et al., 1993).

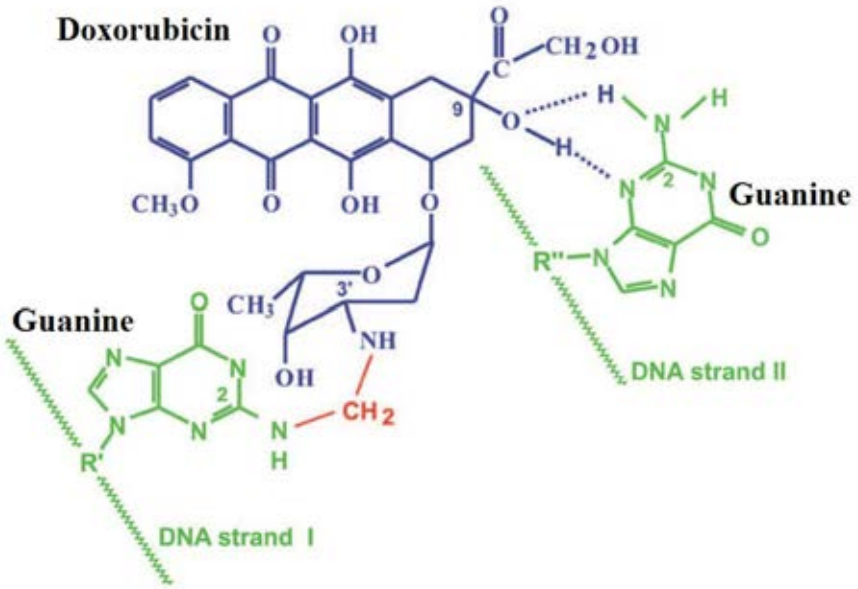


Figure 2. The interaction between doxorubicin and guanine base of double strand DNA (Cutts et al., 2005).

1.3 ROS generation

Recently, the capability of doxorubicin to generate ROS has been depicted in many studies. Therefore the influence of oxidative stress on doxorubicin-induced toxicity is continuously investigated (Gilliam et al., 2011; Granados-Principal et al., 2010; Luanpitpong et al., 2012; Mizutani et al., 2005; Wang et al., 2004). Superoxide anion ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) are remarkably accepted as ROS involving in cytotoxicity of doxorubicin in cancer and non-cancer cells. Both $O_2^{\bullet-}$ and H_2O_2 can deteriorate mitochondrial membrane resulting in cytochrome *c* releasing, activation of caspase proteins and apoptotic cell death, respectively (Green and Leeuwenburgh, 2002; Luanpitpong et al., 2012; Mizutani et al., 2005; Tsang et al., 2003; Wang et al., 2004). The demolition of these ROS efficiently prevents doxorubicin-induced apoptosis (Granados-Principal et al., 2010). Although doxorubicin induces the production of ROS in both cancer and non-cancer cells, the protective effect of antioxidant seems to be dominate in normal cells. This implies that cytotoxicity of doxorubicin in non-cancer cells undergoes mainly in ROS-related mechanism (Wang et al., 2004).

Doxorubicin largely initiates ROS through a redox-cycling system. The redox-cycling pathway of doxorubicin begins with the formation of a free radicle molecule of semiquinone by NADPH (Figure 3). After penetration into cytoplasm, doxorubicin will receive one electron form NADPH by catalyzing of cytochrome P450 reductase. This reduced doxorubicin or semiquinone free radical is unstable and rapidly reacts with oxygen molecule (O_2) resulting in free radical of $O_2^{\bullet-}$. After donating electron to oxygen, the semiquinone is converted into parental molecule of doxorubicin which

further generates a new redox-cycling thus small amount of doxorubicin can proceed many cycle of redox reaction. If oxygen (O_2) is not present, the semiquinone free radical will loss daunosamine sugar molecule and generate free radical at C7 of anthraquinone ring.

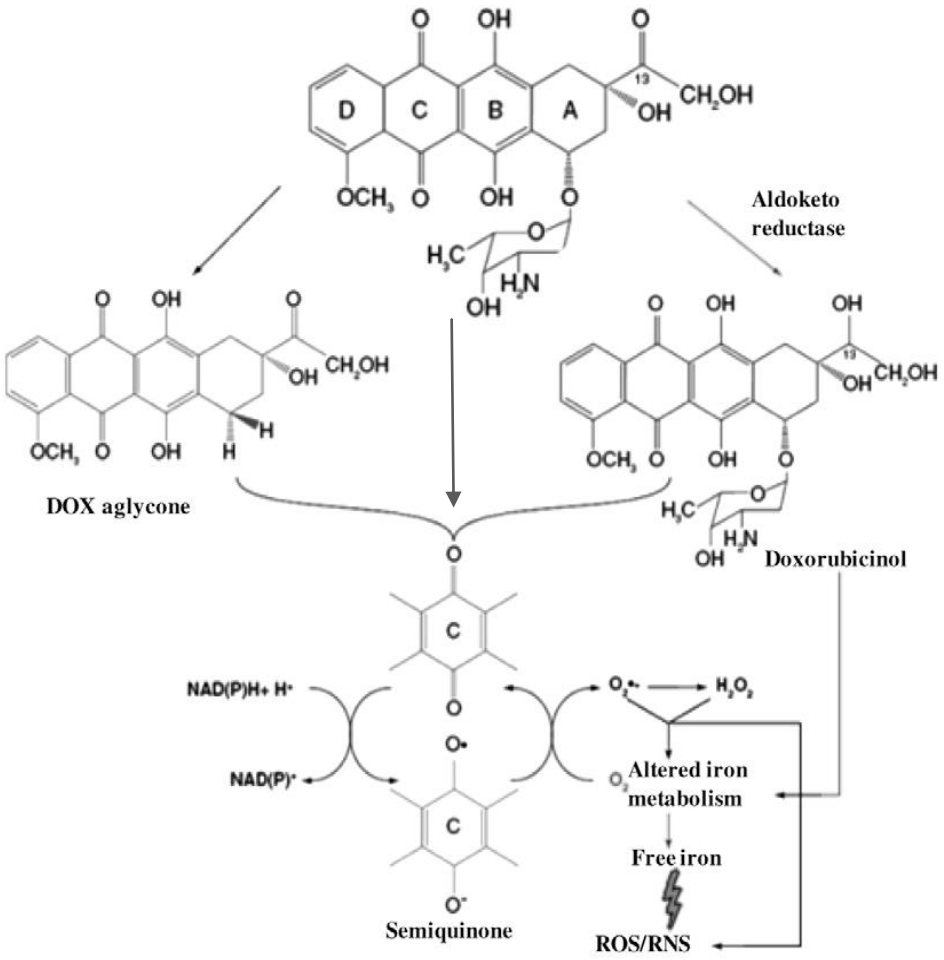


Figure 3. Pathway of doxorubicin-generated reactive oxygen species. In cellular cytoplasm, doxorubicin is metabolized into doxorubicinol and 7-deoxyaglycone (DOX aglycone). All of doxorubicin, 7-deoxyaglycone and doxorubicinol can enter the redox-cycling system to generate free radical of superoxide anion (Granados-Principal et al., 2010; Riddick et al., 2005).

This C7 free radical like other ROS can instantly damage DNA and cellular proteins (Keizer et al., 1990; Riddick et al., 2005). Doxorubicin-Fe^{2+} complex is the product of direct reaction between doxorubicin and cellular iron molecule. Doxorubicin-Fe^{2+} complex induce ROS formation through transformation of O_2 and H_2O_2 into $O_2^{\bullet-}$ and hydroxyl radical ($OH^{\cdot}$), respectively. Another pathway of doxorubicin-induced ROS is agitation of iron metabolism. Doxorubicin is metabolized by aldoketon reductase resulting in doxorubicinol which disturb cytoplasmic iron level consequence with free iron leakage and oxidative stress (Granados-Principal et al., 2010).

1.4 The influence of Mcl-1, an anti-apoptotic protein

Apoptosis is a programed cell death that functionally gets rid of harmful or unusable cell in order to maintain homeostasis and cell population (Elmore, 2007). There are specific biochemical and morphology changes during apoptotic process. After stimulation by apoptotic trigger such as cytotoxic agent, ROS and the imbalance between pro-apoptotic and anti-apoptotic (survival) protein, the integrity of mitochondrial membrane will be affected and lead to leakage of cytochrome *c*. Cytochrome *c* will activate caspase cascade to destroy cellular protein (Saraste and Pulkki, 2000). The morphological characters occur subsequently following these biochemical changes. The degradation of cellular protein results in shrinking of apoptotic cell following with fragmenting of condensed DNA and blebbing of apoptotic body (Ouyang et al., 2012). Because apoptosis cause damage only triggered cell without induction of inflammation to surround tissue, many antitumor drugs including doxorubicin aim to urge apoptotic cell death in the target cell (Elmore, 2007).

Although doxorubicin mediates apoptosis through various possible pathways, the role of Bcl-2 family proteins in sensitization or resistance to doxorubicin-induced toxicity has been widely elucidated. The suppression of Mcl-1, a Bcl-2 anti-apoptotic protein, significantly increases doxorubicin-induced apoptosis in neuroblastoma and leukemia cells (Lestini et al., 2009; Lopez-Royuela et al., 2010). Meanwhile, the overexpression of Mcl-1 sufficiently prevents apoptotic cell death initiated by doxorubicin in both *in vivo* and *in vitro* model (Bolesta et al., 2012; Katoh et al., 1998; Lestini et al., 2009; Wei et al., 2001; Wirth et al., 2005; Yang et al., 2012). Unfortunately, heart and renal tissues expresses low to moderate level of Mcl-1 (Krajewski et al., 1995). Therefore, Mcl-1 protein seems to be not sufficient in manipulating the toxicity of doxorubicin especially in renal cells.

2. Doxorubicin-induced renal toxicity

Despite the great benefit of antitumor activity, administration of doxorubicin is restricted due to its undesired side-effects of cardiomyopathy and renal failure (Mansour et al., 1999; Saad et al., 2001; Singal and Liskovic, 1998). After intravenous injection, doxorubicin is metabolized into an active molecule, doxorubicinol, via NADPH-dependent aldoketoreductase enzymes mostly in liver and renal cell (Figure 3). Both doxorubicin and doxorubicinol are eliminated through bile and urine within 7 days after administration. Evaluation on drug distribution has revealed that doxorubicin concentration is usually higher in organ tissue such as heart and kidney rather than in plasma (Speth, van Hoesel, and Haanen, 1988). The monitoring of renal function has been suggested after revelation of high level of serum creatinin and deterioration of renal epithelial cells in patient administered with

doxorubicin (Burke et al., 1977). Various *in vivo* and *in vitro* studies have demonstrated that doxorubicin causes apoptosis in tubular renal cells (Cheng et al., 2006; Ueda and Shah, 2000; Zhang et al., 1996). Although the underlying mechanisms remain unclear, the generation of ROS and deletion of cellular antioxidant mechanisms are presented in doxorubicin-induced nephrotoxicity (Kaur et al., 2012). These evidences have been suggested by experiments using with many chemical and natural antioxidants that effectively protect renal cell death induced by doxorubicin both *in vitro* and *in vivo* (Boonsanit et al., 2006; Boutabet et al., 2011; Chen et al., 2006; Lebrecht et al., 2004).

Mcl-1, a pro-survival protein

Mcl-1 or myeloid cell leukemia-1 is one of the Bcl-2 family proteins that consist of pro-survival and pro-apoptotic proteins. Mcl-1 including Bcl-2, Bcl-xL, Bcl-w, and A1 are categorized into Bcl-2 anti-apoptotic proteins, while the members of Bcl-2 pro-apoptotic proteins are Bax, Bak, Bim and Bok/Mtd (Youle and Strasser, 2008). All of the Bcl-2 family proteins are comprised of BH homologous domain. There are four different BH structures including BH1, BH2, BH3 and BH4. All of these four BH domains and transmembrane region are mostly presented in Bcl-2 anti-apoptotic protein but not in Mcl-1. Although Mcl-1 lacks of BH4 domain, it contains 350 amino-acids which are larger than other Bcl-2 anti-apoptotic protein. In place of BH4, Mcl-1 is added with two polypeptides of proline, glutamic, serine and threonine amino acid (PEST domain) (Le Gouill et al., 2004). The localization of Mcl-1 is mainly found at the mitochondrial membrane, although it is also present in the

cytoplasm and membrane of nuclease and endoplasmic reticulum (Figure 4) (Michels et al., 2005). Despite their similar function of anti-apoptosis, there is a distinction of tissue and cellular distribution between Mcl-1 and Bcl-2. Mcl-1 is highly expressed in differentiated cells which are mostly located at the epithelial layer. Meanwhile Bcl-2 is depicted in a less differentiated and self-renewing cell at lower basement area. High accumulation content of Mcl-1 is found in the tissue organs of skin, testis and adrenal. For other tissues including renal, heart, lung and stomach, rare to low detection of Mcl-1 level has been reported (Krajewski et al., 1995). Comparison between the normal and cancer tissue, Beroukhim and co-workers have revealed the increase of Mcl-1 protein in human cancer specimens. This incident explains the observed long survival rate of tumor cells (Beroukhim et al., 2010).

Mcl-1 displays various biological functions in different cell types, including embryogenesis and differentiation of lymphocyte. In terms of tumorigenic, Mcl-1does not increase proliferation of cancer cells but plays a role in maintaining the cancer cell population through inhibition of apoptosis (Michels et al., 2005; Thomas et al., 2010). Mcl-1 prevents the disruption of mitochondrial membrane which is stimulated by apoptotic triggers. The activation of apoptosis mediates the oligomerization of Bcl-2 pro-apoptotic proteins, Bax and Bak, resulting in a formation of pores on the mitochondrial membrane. At survival condition, both mitochondrial and cytoplasmic Mcl-1 bind with Bcl-2 pro-apoptotic proteins, Bak and Bim, as well as BH-3 only apoptotic proteins, tBid and Puma, in order to prevent the oligomerization of Bax and Bak on the mitochondrial membrane (Figure 4). However, sequestration of Bcl-2 pro-apoptotic protein from Mcl-1 via competitive binding by Noxa can be stimulated after the apoptotic activation or depletion of Mcl-1. Free Bax and Bak then oligomerize to

form a pore on mitochondrial membrane. After the leakage of cytochrome *c* from mitochondria through pore of Bax and Bak, caspase cascade and apoptosis are activated, respectively (Akgul, 2009; Thomas et al., 2010).

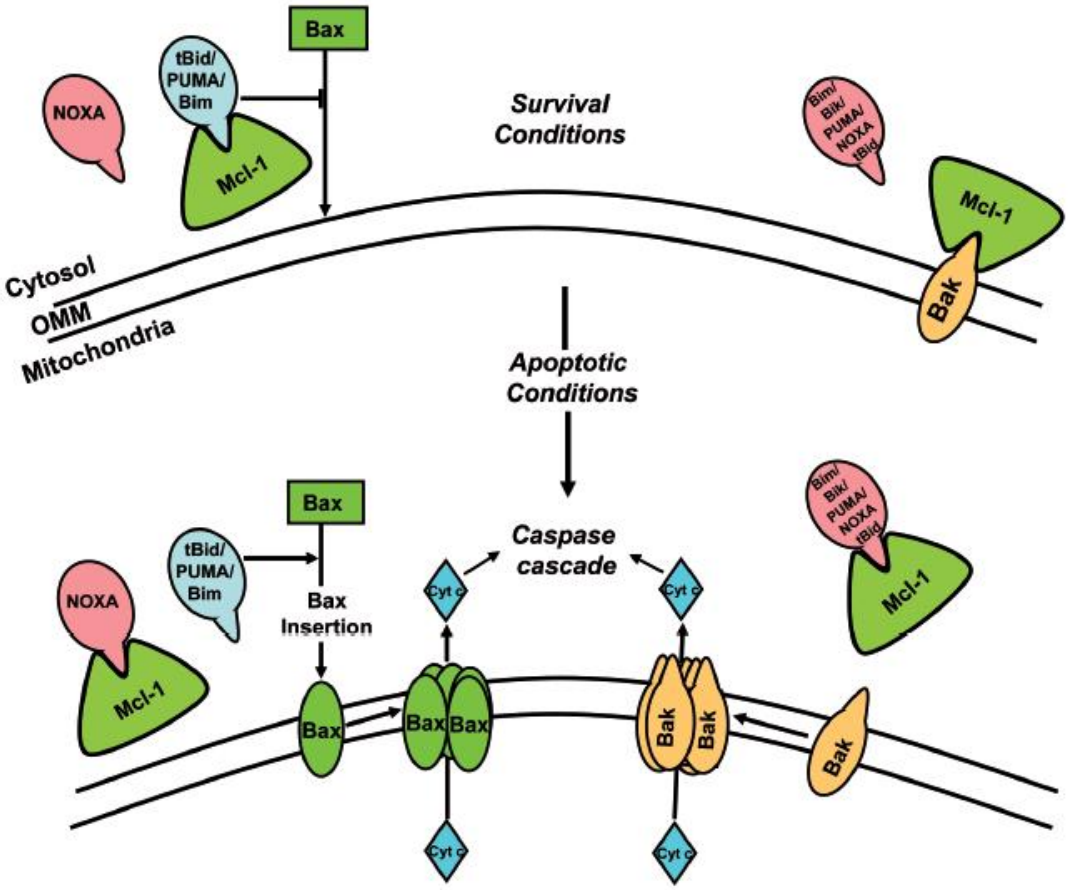


Figure 4. Localization and role of Mcl-1 in both survival and apoptotic condition (Akgul, 2009).

Down regulation of Mcl-1 without alteration of Bcl-2 level has been notified in apoptosis induced by various toxic agents including aspirin, staurosporin and cisplatin. These reagents decrease Mcl-1 via inhibition of transcription and translation as well as stimulation of degradation (Iglesias-Serret et al., 2003; Yang et al., 2007).

Mcl-1 is a rapid turnover protein with half-life about 1 to 3 hour. PEST domains are responsible for the stability of Mcl-1. The regulation of Mcl-1 degradation has been continuously clarified. Like other cellular proteins, degradation of Mcl-1 is described through ubiquitination and proteosome degradation pathway. However, during apoptosis, caspase3 also break Mcl-1 into a pro-apoptotic protein (Figure 5) (Michels et al., 2005).

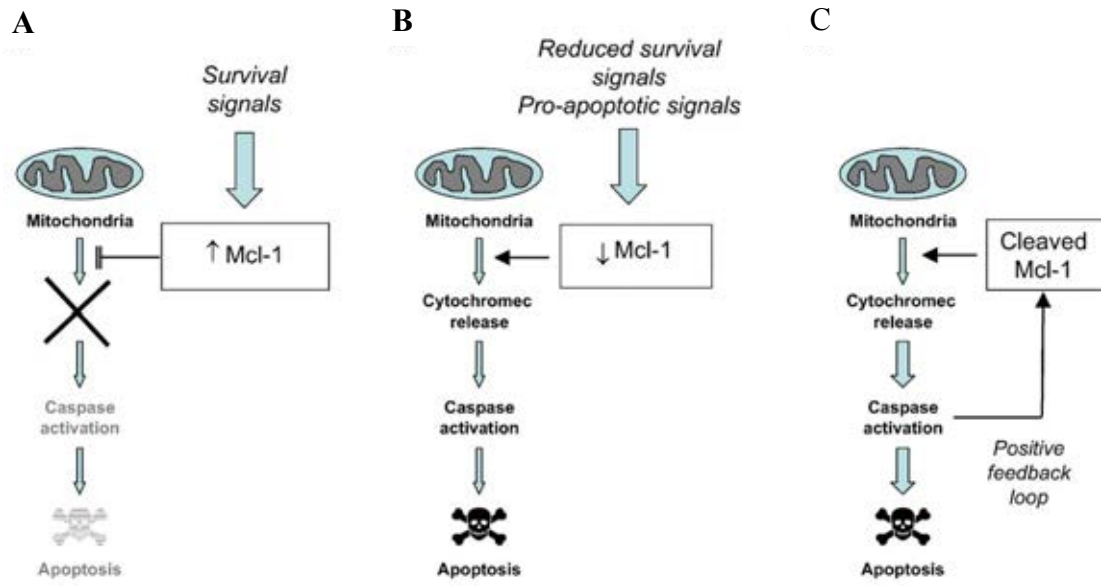


Figure 5. The overview relation between Mcl-1 and apoptotic cell death. (A) Increase of Mcl-1 by survival signals prevents apoptosis. (B) Depletion of Mcl-1 activates apoptotic cell death. (C) Caspase-cleaved Mcl-1 generates another loop of apoptosis (Michels et al., 2005).

Recently, the involvement between Mcl-1 level and ROS has been evaluated through many studies. Blatt, et al. have demonstrated the degradation of Mcl-1 mediated by superoxide anion ($O_2^{\bullet -}$). Reactive oxygen species (ROS) might activate

the ubiquitination of Mcl-1 by E3 ubiquitin ligase Mule following with recognition and degradation by proteasome (Blatt et al., 2009). ROS and downstream JNK are also indicated in the phosphorylation of Mcl-1 at PEST motif resulting in the inactivation and reduction of Mcl-1 level (Chiu et al., 2012; Inoshita et al., 2002; Jang et al., 2010; Kang et al., 2008). The depletion of Mcl-1 in the oxidative stress condition has been shown to be recovered by an effective antioxidant (Chiu et al., 2012; Jang et al., 2010; Kang et al., 2008).

Reactive oxygen species (ROS)

Cellular oxidative stress is a circumstance that involves with high amount of oxidizing agent, especially reactive oxygen species (ROS). For various diseases including Alzheimer's, ischemic/reperfusion injury, cancer, hypertension, and diabetes, the increase of intracellular ROS can cause the damage on biological molecules such as protein, lipid, and DNA and always end with cell death (Winyard, Moody, and Jacob, 2005). ROS can be described as oxygen-derived free radicals such as superoxide anion ($O_2^{\bullet -}$), hydroxyl radical ($OH^{\bullet}$), peroxyl ($RO_2^{\bullet}$), and alkoxyl ($RO^{\bullet}$) as well as oxygen-derived non-radical species such as hydrogen peroxide (H_2O_2) (Circu and Aw, 2010).

ROS are generally generated during cellular metabolic pathway even at the normal condition. At low or moderate concentrations, ROS act like a protective molecule or cellular signaling system for physiological benefits such as infectious defense molecules, but the unbalance between ROS formation and cellular antioxidant mechanisms leads to cellular injury and cell death (Figure 6) (Nordberg and Arner,

2001; Valko et al., 2007). The leakage of electron from mitochondrial electron transport chain to oxygen molecule (O_2) via Complex I and III is the initiation of ROS formation, particularly $O_2^{\bullet-}$. Although superoxide anion ($O_2^{\bullet-}$) cannot escape from mitochondrial membrane because of highly ionic charge, it is also found in cytoplasm as a product from the reaction between O_2 and flavonenzyme such as xanthine oxidase (Nordberg and Arner, 2001). Both mitochondrial and cytoplasmic $O_2^{\bullet-}$ are dismutated into H_2O_2 by manganese superoxide dismutase (Mn-SOD) and copper/zinc superoxide dismutase enzyme (Cu/Zn-SOD), respectively. Highly reactive ROS, $OH^{\bullet}$ is an end product of Fenton reaction, the catalytic reaction between H_2O_2 and ferrous (Fe^{2+}) or cupric (Cu^{+}). Superoxide anion ($O_2^{\bullet-}$) also participates in Haber-Weiss reaction in order to generate $OH^{\bullet}$. Indeed, Haber-Weiss reaction is the combination of Fenton reaction and the reduction of ferric (Fe^{3+}) or cuprous (Cu^{2+}) by $O_2^{\bullet-}$ (Nordberg and Arner, 2001; Valko et al., 2007).

Fenton reaction: $H_2O_2 + Fe^{2+}/Cu^{+} \longrightarrow OH^{\bullet} + OH^{-} + Fe^{3+} / Cu^{2+}$

Reduction of metal ions: $O_2^{\bullet-} + Fe^{3+} / Cu^{2+} \longrightarrow O_2 + Fe^{2+}/Cu^{+}$

Haber-Weiss reaction: $H_2O_2 + O_2^{\bullet-} \longrightarrow OH^{\bullet} + OH^{-} + O_2$

ROS defensive mechanisms

Cellular defensive mechanisms against oxidative stress can be divided into non-enzymatic and enzymatic antioxidant (Valko et al., 2007).

1. Non-enzymatic antioxidant

Ascorbic acid, α-tocopherol, lipoic acid, reduced glutathione (GSH) and ubiquinone are a number of low molecular weight antioxidant compounds. These non-enzymatic antioxidants can get rid of ROS by themselves or cooperate with other molecules. Vitamin E or α-tocopherol reacts with peroxyl radical ($RO_2^{\bullet}$) in order to stop the chain reactions of lipid peroxidation. Meanwhile, ascorbic acid and GSH act as oxidizing agent to reactivate an α-tocopherol free radical which is a result from its ROS scavenging activity. Additionally, GSH with catalyzing of enzymatic antioxidant can scavenge H_2O_2 (Forman, Zhang, and Rinna, 2009; Valko et al., 2007).

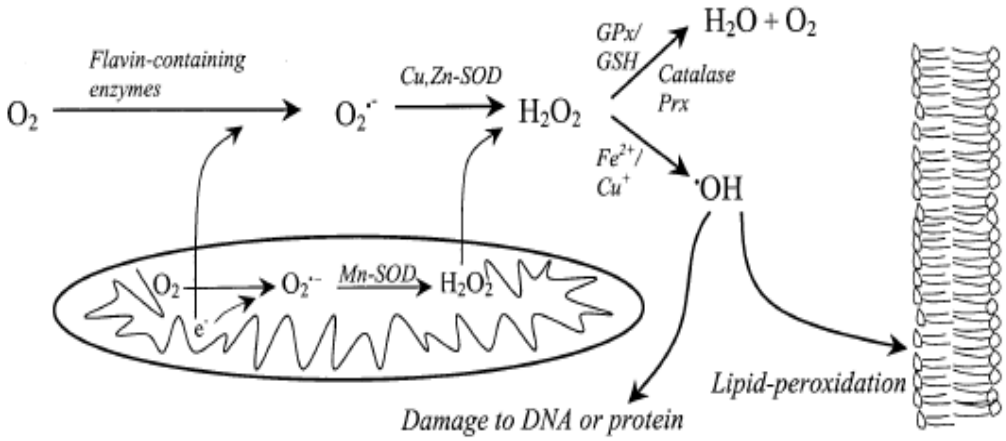


Figure 6. Cellular ROS production. Superoxide anion ($O_2^{\cdot -}$), an ROS precursor, is generated in mitochondria and cytoplasm. Superoxide dismutase (SOD) converts $O_2^{\cdot -}$ into hydrogen peroxide (H_2O_2). Metal ions of Fe^{2+} and Cu^{+} then rapidly catalyze the formation of hydroxyl radical ($OH^{\cdot}$) from (H_2O_2) (Nordberg and Arner, 2001).

GSH is the largest amount of non-enzymatic antioxidant in cellular system. It is a tripeptide of three amino acid, cysteine, glutamate and glycine (Forman et al., 2009). ATP dependent glutamate cysteine ligase (GCL) or γ-glutamylcystein synthase (γ-GCS) catalyzes the conjugation of the peptide bond between cysteine and glutamate to generate γ-glutamylcysteine molecule (Figure 7). Because the production of γ-glutamylcysteine is a critical step in GSH synthesis, cysteine and γ-glutamylcystein synthase (γ-GCS) are considered as rate-limiting substrate and rate-limiting enzyme, respectively. Finally, glycine is added into γ-glutamylcysteine by glutatnione synthase (GSH synthase) resulting in GSH molecule (Forman et al., 2009; Rahman and MacNee, 2000). In spite of the production in cytoplasm, GSH can be found in extracellular fluid. The average amount of the intracellular GSH is about 1 to 2 mM, except for hepatic cell which can be as high as 10 mM. Liver is an organ that highly produces and exports GSH to other organs including lung, heart, brain and kidney. Due to its high expose to oxidative stress, renal cell has capability to directly transport external GSH into cytoplasm or through breakdown GSH into dipeptide by γ-glutamyl transpeptidase enzyme (Deneke and Fanburg, 1989).

GSH is used for eradication of H_2O_2 via enzymatic pathway of glutathione peroxidase (Figure 8). However, GSH can directly scavenge free radical of $O_2^{\bullet-}$ and $OH^{\cdot}$ without the catalyzation from other molecules. The reaction between GSH and $OH^{\cdot}$ results in water and thiyl radical ($GS^{\cdot}$). This thiyl radical can react with another molecule of $GS^{\cdot}$ or GSH to form glutathione disulfide (GSSG) or glutathione disulfide radical ($GSSG^{\cdot-}$), respectively. At the presence of oxygen molecule (O_2), $O_2^{\bullet-}$ is generated via the interaction between $GSSG^{\cdot-}$ and O_2 (Arteel and Sies, 2001). Asada

and Kanemutsu have shown that GSH can directly convert $O_2^{\bullet-}$ into H_2O_2 under the condition of physiological pH (Asada and Kanematsu, 1976).

GSH scavenge $OH^{\bullet}$: $GSH + OH^{\bullet} \longrightarrow H_2O_2 + GS^{\cdot}$ (1)

: $GSH + GS^{\cdot} \longrightarrow H^{+} + GSSG^{\cdot}$ (2)

: $GSSG^{\cdot} + O_2 \longrightarrow O_2^{\bullet-} + GSSG$ (3)

GSH scavenge $O_2^{\bullet-}$: $GSH + O_2^{\bullet-} + H^{+} \longrightarrow H_2O_2 + GS^{\cdot}$

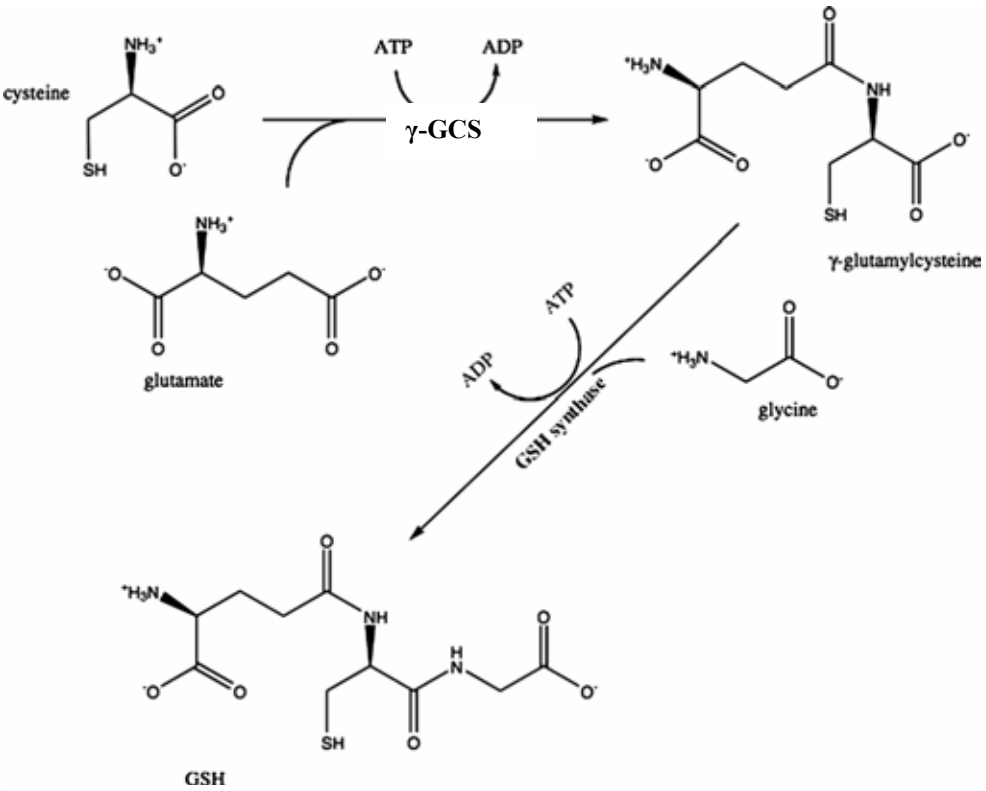


Figure 7. Glutathione (GSH) synthetic scheme. Cysteine and γ-glutamylcystein synthase (γ-GCS) play a crucial role in the rate limiting step of glutathione synthesis (Forman et al., 2009).

2. Enzymatic antioxidant

2.1 Superoxide dismutase (SOD)

Superoxide dismutase (SOD) in mammalian cells can be mainly categorized into two types, Cu/Zn-SOD in cytoplasm and Mn-SOD in mitochondria (Nordberg and Arner 2001). The catalytic reaction between $O_2^{\cdot-}$ and SOD consists of the oxidation and reduction at metal (M) center of SOD.

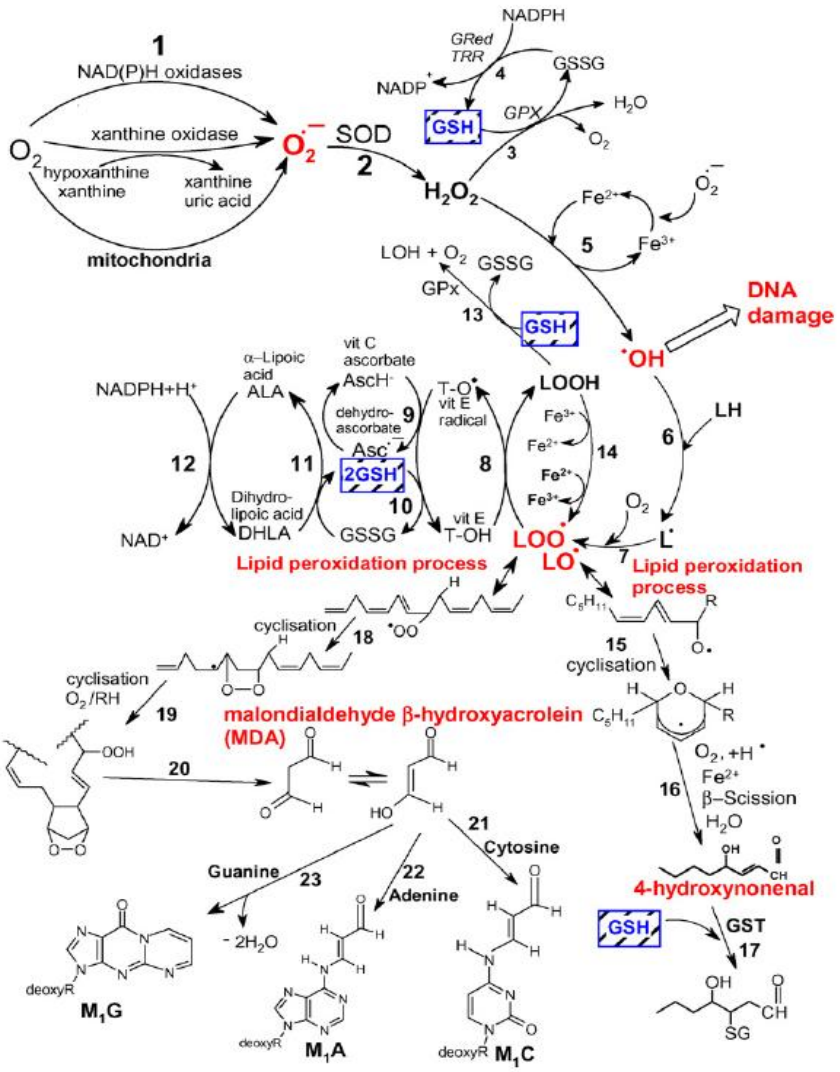


Figure 8. Summary scheme of ROS generation, ROS-induced cell damage and cellular antioxidant pathway (Valko et al., 2007).

Briefly, two molecules of $O_2^{\bullet-}$ are dismutated into one molecule of H_2O_2 and O_2 as shown below (Abreu and Cabelli, 2010). Superoxide anion ($O_2^{\bullet-}$) is continuously generated from the mitochondrial respiratory chain, and it acts as a precursor for generation of other harmful ROS. Therefore, the efficacy of SOD on the inhibition of the progression of ROS-related disease such as reversible fibrosis and graft rejection has been demonstrated (McCord and Edeas, 2005). This indicates the possibility to use SOD as an antioxidant therapy.

$$M^{(n+1)+} + O_2^{\bullet-} \longrightarrow M^{n+} + O_2 \quad (1)$$

$$M^{n+} + O_2^{\bullet-} + 2H^+ \longrightarrow M^{(n+1)+} + O_2 + H_2O_2 \quad (2)$$

Overall: $2O_2^{\bullet-} + 2H^+ \longrightarrow O_2 + H_2O_2$

2.2 Catalase

Catalase localizes predominantly in a cytosolic organelle, peroxisomes. Catalase can detoxify H_2O_2 and other substrates such as alcohol and phenol (Nordberg and Arner, 2001). Similar to superoxide dismutase, catalase interacts with H_2O_2 through the redox reaction of ferriprotoporphyrin IX at the active site (Kirkman and Gaetani, 2007). The equation of interaction between catalase and H_2O_2 is indicated as following: $2H_2O_2 \xrightarrow{\text{Catalase}} O_2 + 2H_2O$.

2.3 Glutathione redox systems

Although glutathione (GSH) is synthesized only in cytoplasm, it can be found in the mitochondria and nuclease. Most of cellular GSH act as electron donor to H_2O_2 and other peroxides (ROOH) including lipid-OOH (Figure 9). Depending on the enzyme glutathione peroxidase (GPx), two molecules of GSH convert one molecule of H_2O_2 into water. After losing the electron, two molecule of glutathione will

produce the oxidized form of glutathione, GSSG (Deneke and Fanburg, 1989; Forman et al., 2009). Glutathione disulphide (GSSH) is then reactivated back to GSH by NADPH-dependent flavonenzyme glutathione reductase (GR). Additionally, GSH has been shown to directly scavenge ROS through the interaction with H_2O_2, $O_2^{\bullet-}$ and free radical of vitamin C (Nordberg and Arner, 2001; Valko et al., 2007).

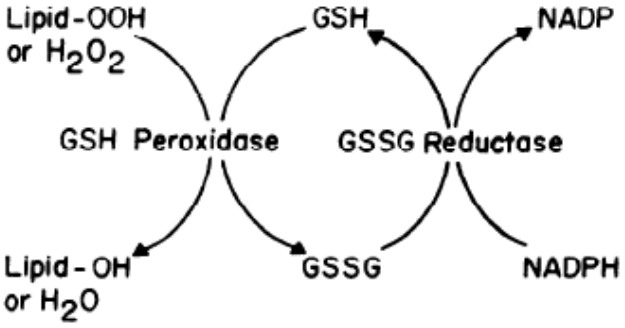


Figure 9. Glutathione redox system (Deneke and Fanburg, 1989).

Plaunotol

Plaunotol or (2E,6Z,10E)-7-hydroxymethyl-3,11,15-trimethyl-2,6,10,14-hexadecatetraen-1-ol (Figure 10) is an acyclic diterpene which can be extracted from leaves of Thai medicinal plant, Plau-noi (C*roton stellatopilosus* Ohba.). In the past, leave of plau-noi were used as traditional remedy for gastric ulcer and topical antibacterial but nowadays, plaunotol extracts has been developed to become a major active ingredient of the commercial drug named Kelnac®. Although plau-noi is widely grown in Thailand, Kelnac® is registered and manufactured in Japan for treatment of gastric and peptic ulcer (Kaneko et al., 1995; Paduch et al., 2007; Wada et al., 1997).

Plaunotol has been shown to inhibit gastritis through many mechanisms. Cytoprotective activity of plaunotol is the result from the increasing of prostaglandin E2 (PGE2), an important molecule for controlling gastric mucosal barrier (Ushiyama et al., 1987). Plaunotol activates NF-κB transcriptional factor to up-regulate the expression of cyclooxygenase2 (COX2) which sequentially increases the secretion of PEG2 (Fu et al., 2005). The inhibitory effect on *Helicobacter pylori* (*H.pylori)* bacteria and the capability to reduce reactive oxygen species (ROS) formation also are proposed for anti-gastritis mechanisms of plaunotol (Takagi et al., 2000).

A

B

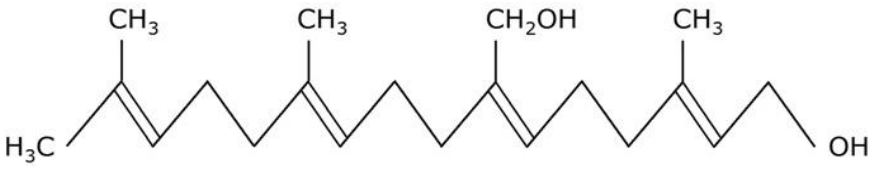


Figure 10. (A) Plau-noi leaves (B) Isoprenoid structure of plaunotol (Yoshikawa et al., 2009).

H.pylori is an important factor on recurrence of gastric and peptic ulcer. Combination regimen of plaunotol with clarithomycin or amoxicillin effectively gets

rid of *H.pylori* both *in vitro* and *in vivo* (Koga et al., 2002). ROS especially in leukocytes are involved in the gastric and duodenal mucosal damage by non-steroidal anti-inflammatory drugs (NSAIDs) (Matsui et al., 2011). Plaunotol has been demonstrated to inhibit gastric mucosal injury by NSAIDs through the reduction of neutrophil's ROS production (Murakami et al., 1999). From clinical study, seven days of plaunotol regimen significantly reduced superoxide formation as shown by chemiluminescent response in whole blood cells of smoking volunteers (Okabe et al., 1995). The antioxidant property of plaunotol has been reported again as a possibly important pathway for inhibition of acute gastritis progression in rat (Ohta et al., 2005). Although plaunotol has been shown for antioxidant activity *in vivo* but the underlying mechanisms are still unknown.

According to high distribution into renal tissue, safety profile in human, and antioxidant activity, plaunotol shows a prospering possibility to be developed as a renopreotective drug. Moreover, the investigation on a novel pharmacological activity of plaunotol will bring a valuable opportunity for Thai pharmaceutical industry to produce a healthcare product from Thai plant extract.

CHAPTER III

MATERIALS AND METHODS

Materials

1. Cell culture

Human proximal tubular epithelial renal (HK-2) cells were obtained from the American Type Culture Collection, ATCC (Manassas, VA, USA). HK-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Gaithersburg, MA, USA) supplemented with 2 mM L-glutamine, 10% fetal bovine serum (FBS), and 100 units/mL of penicillin/streptomycin (Gibco, Gaithersburg, MA, USA) in 5% CO_2 at 37 °C.

2. Chemical reagents

Standardized plaunotol extract with 95% purity was a generous gift from Thai Sankyo Co., Ltd presently taken by Tipco Foods (Thailand) PCL. Doxorubicin and Manganese (III) Tetrakis (4-Benzoic Acid) Porphyrin chloride (MnTBAP) were purchased from Calbiochem (San Diego, CA, USA). 2,3-Dimethoxy-1,4-naphthoquinone (DMNQ), Iron(II) sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$), 3% w/w hydrogen peroxide (H_2O_2) solution, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), N-acetylcysteine (NAC), reduced glutathione (GSH), Hoechst 33342, propidium iodide (PI), dimethysulfoxide (DMSO), fluorescein, 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH), 2,2-

diphenyl-1-picrylhydrazyl (DPPH), and monochlorobimane (MCB) were obtained from Sigma Chemical, Inc. (St. Louis, MO, USA). 2',7'-Dihydrodichlorofluorescein diacetate (H_2DCF-DA) and dihydroethidium (DHE) were obtained from Molecular Probes (Eugene, OR, USA). Hydroxyphenyl fluorescein (HPF) was obtained from Cell Technology (Mountain View, CA). pcDNA3.1-hMcl-1 plasmid was purchased from Addgene (Cambridge, MA, USA). pcDNA3.1 plasmid and lipofectamine 2000 were obtained from Invitrogen (Carlsbad, CA, USA). Antibody for Mcl-1, Bcl-2, MnSOD (Manganese superoxide dismutase), Cu/ZnSOD (Copper-zinc superoxide dismutase), catalase, β-actin as well as HRP secondary antibody were obtained from Abcam (San Francisco, CA, USA). Primary antibody of glutathione peroxidase (GPx1/2) was purchased from Santa Cruze Biotechnology (Dallas, TX, USA).

3. Transient transfection

HK-2 cells were transfected either with pcDNA3.1-hMcl-1 or pcDNA3.1 plasmids using Lipofectamine 2000 as recommended by the manufacturer. After 36 h, the transfected cells were used in the experiment (Dash et al., 2011).

Methods

1. Sample preparation

Doxorubicin was dissolved with water and kept at 4 ºC as a stock solution of 2 mM. Plaunotol was freshly prepared in each experiment by dissolving in 99.5%

ethanol. The final concentration of ethanol in cell culture medium was not more than 0.25%.

2. Cytotoxicity assay

Approximately 10^4 cells/well of HK-2 were seeded in 96-well plate and incubated with fresh culture medium containing growth supplements. After 24 h, the treatments at indicated concentration were added for the indicated time. MTT assay was used for examination of cell viability according to manufacturer instruction. Briefly, 0.5 mg/mL of MTT solution was added to each well and incubated at 37 ºC for 4 h. The formazan crystals which were generated from MTT by succinate dehydrogenase from viable mitochondria were extracted using DMSO. The absorbance of the formazan color was detected by an ELISA microplate reader, Anthros (Durham, NC, USA) at 570 nm. The cell viability was calculated from optical density (OD) reading and represented as ratio of the non-treated control value in the term of percentage cell viability.

$$\% \text{ Cell viability} = \frac{OD_{570}\ \text{treatment}}{OD_{570}\ \text{control}} \times 100$$

3. Apoptotic and necrotic cell death assay

Hoechst 33342 and propidium iodide (PI) co-staining was used to detect mode of cell death. After specific treatments, HK-2 cells were stained with 10 μM Hoechst 33342 and PI 5 μg/mL for 30 min at 37°C. The apoptotic cells with condensed chromatin and/or fragmented nuclei were stained by Hoechst 33342 while PI positively stained necrotic cells. Mode of cell death were visualized and scored under a fluorescence microscope (Olympus IX51 with DP70).

4. Western blot analysis

After incubation with treatment for indicated time, HK-2 cells were placed in lysis buffer (Tris-HCl; pH 7.5, 1% Triton X-100, 150 mmol/L NaCl, 10% glycerol, 1 mmol/L Na_3VO_4, 50 mmol/L NaF, 100 mmol/L phenylmethylsulfonyl fluoride) containing a protease inhibitor cocktail at 4 ºC for 30 min. Total protein was determined from the lysate by the Bradford assay (Bio-Rad Laboratory; Hercules, CA, USA). Equal amount of protein was resolved under denaturing conditions by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto the nitrocellulose membranes (Bio-Rad). The membrane was blocked by 5% nonfat dry milk in TBST containing 25 mmol/L Tris-HCl; pH7.4, 125 mmol/L NaCl, 0.05% Tween 20, and incubated with primary antibodies at 4 ºC for 10 h. Membrane was washed twice with TBST for 10 min, and incubated with horseradish peroxidase-conjugated isotype specific secondary antibodies for 1 h at room temperature. The immune complexes were detected by enhanced chemiluminescence detecting system (Amersham Bioscience; Piscataway, NJ, USA). The quantitative analysis was examined by analyst/PC densitometry software (Bio-Rad).

5. Cellular ROS detection

The formation of reactive oxygen species (ROS) within HK-2 cells was detected by the fluorescence probes, H_2DCF-DA, DHE and HPF. H_2DCF-DA is a probe for general ROS while DHE and HPF are more specific to superoxide anions ($O_2^{\cdot -}$) and hydroxyl radicals ($OH^{\cdot}$), respectively (Brandes and Janiszewski, 2005; Gomes, Fernandes, and Lima, 2005; Sharikabad et al., 2001). After indicated time of treatment, the cells were washed with PBS then incubated with 10 μg/mL

fluorescence probe in serum free medium at 4ºC for 30 min. Fluorescence intensity of DCF (excitation wavelength, 488 nm; emission wavelength, 538 nm), DHE (excitation wavelength, 488 nm; emission wavelength, 610 nm) and HPF (excitation wavelength, 488 nm; emission wavelength, 520 nm) were examined under fluorescence microplate reader and fluorescence microscope.

6. Diphenyl-picrylhydrazyl (DPPH) assay

Direct free radical scavenging activity of plaunotol (cell-free model) was analyzed by DPPH (2,2-diphenyl-1-picrylhydrazyl hydrate) assay. The violet color of $DPPH^{\cdot}$ will be fade into light-yellow color of DPPH-H after the oxidation by antioxidant compound (Nikolova, 2011). The DPPH solution was freshly prepared by dissolved in methanol (Parejo et al., 2000). After mixing 5 µl of plaunotol solution and 195 µL of 100 µM DPPH solution in 96-well plate, the samples were kept away from light at 37ºC for 30 min. Then the decrease in absorption of $DPPH^{\cdot}$ was measured via ELISA microplate reader at 515 nm. Free radical scavenging activity is calculated by the following formula.

$$\% \text{ Scavenging activity} = \frac{(A_B - A_A) \times 100}{A_B}$$

Where: A_B = absorption of control

A_A = absorption of plaunotol solution

7. Oxygen radical absorbance capacity (ORAC) assay

Capability of plaunotol on direct scavenge peroxyl radical ($ROO^{\cdot}$) was evaluated by ORAC assay. Briefly, mixture between plaunotol and fluorescein in

phosphate buffer (pH7.4) was prepared in 96-well plate and kept at 37 ºC for 10 min. After that a peroxyl radical generator, AAPH (2,2′-Azobis(2-methylpropionamidine) dihydrochloride), was added into the pre-incubated mixture to reduce the intensity of the fluorescent probe. The fluorescent intensity was monitored at 37 ºC every 90 sec for 60 min by using the fluorescent microplate reader at the excitation and emission wavelengths of 485 and 530 nm, respectively. GraphPad Prism software was used for generation of area under the curve (AUC). Graph of the net AUC, subtraction between AUC of sample and blank, and Trolox™ standard concentrations were plotted and then Trolox™ Equivalents were calculated (Zulueta, Maria, and Frígola, 2009).

8. Cellular glutathione (GSH) detection

Monochlorobimane (MCB) can react with reduced glutathione (GSH) forming a fluorescent compound of GSH-bimane through the catalystic reaction of glutathione S-transferees (GST) enzyme (Yang and Tiffany-Castiglioni, 2005). HK-2 cells were seeded in 96-well black clear bottom plate and cultured in the incubator at 37 ºC and 5% CO_2 for 24 h. Plaunotol or N-acetylcysteine (NAC), a substrate for GSH synthesis, then was added into cells' culture medium. After incubation for indicated time, the culture media was removed and the cells were washed twice with PBS. The solution of 40 μM MCB in serum free media was added into each well. After incubation at 37ºC for 2 h, the fluorescent of GSH-bimane product was examine under fluorescent microplate reader at excitation/emission wavelength of 390/460 nm. The relative GSH was calculated as indicated below.

$$\text{Relative GSH} = \frac{(F_{treatment} - F_{blank})}{(F_{control} - F_{blank})}$$

Where: $F_{treatment}$ = fluorescence response of treated cells

$F_{control}$ = fluorescence response of untreated control cells

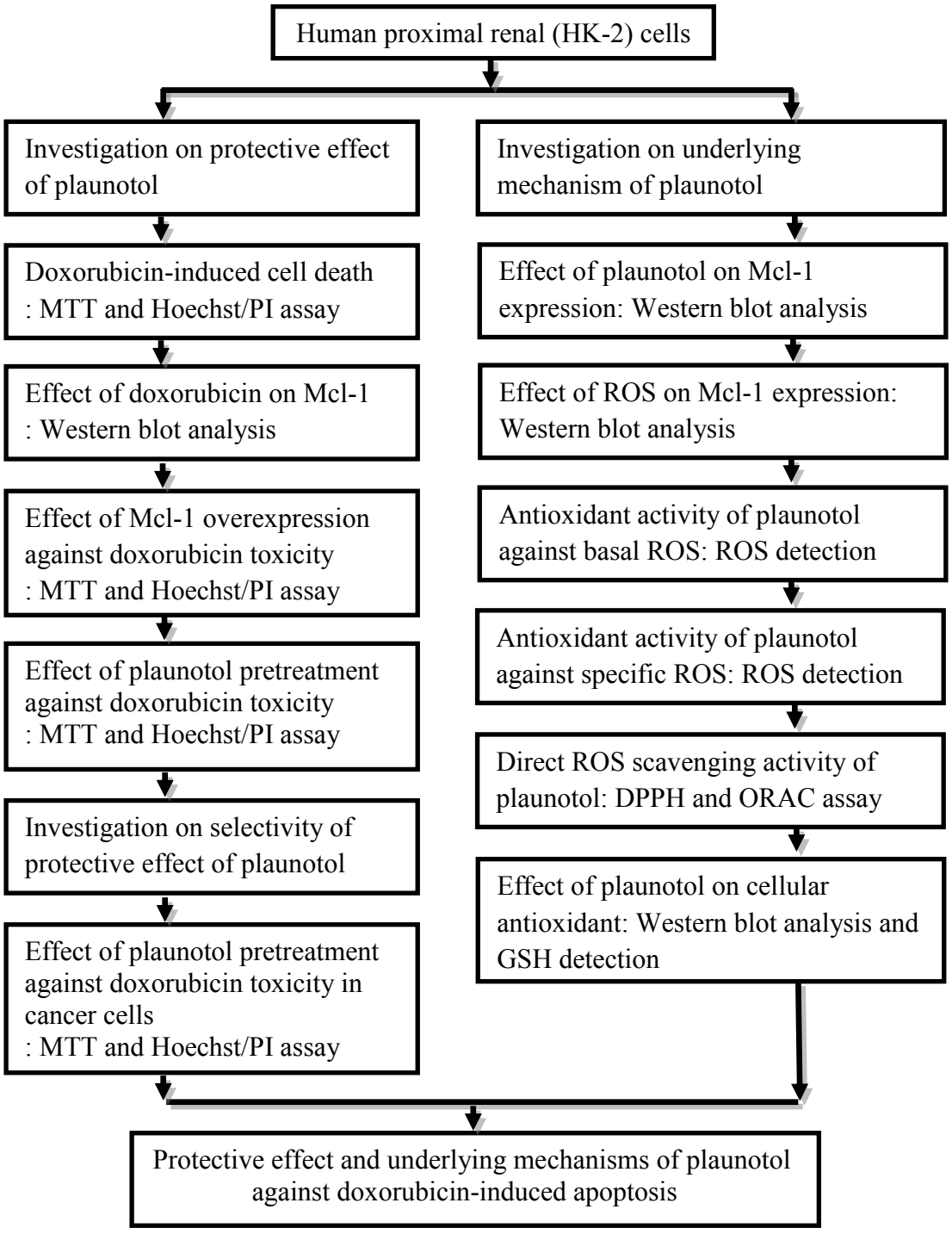
$F_{control}$ = fluorescence response of PBS

9. Statistical analysis

All the experiments were repeated at least three times. Statistical analysis was performed using One-way ANOVA. A *P*-value of less than 0.05 would be considered as statistical significance.

Experimental design

In order to evaluate the protective effect of plaunotol in human renal cells, the investigation was designed as sequential experiments described in the following diagram.



CHAPTER IV

RESULTS

1. Dox-induced toxicity in human renal cells

In order to investigate the possibility of plaunotol in attenuating renal cell toxicity induced by doxorubicin (Dox), the cytotoxicity of Dox was first characterized. Human renal proximal tubular HK-2 cells were treated with various concentrations of Dox up to 8 μM for various time points within 24 h. The resulting cell viability of the treated and non-treated control cells were then verified using MTT viability assay. Figure 11 shows the decrease of cell viability as a result of doxorubicin treatment in dose- and time-dependent manners. The cell viability was observed to be reduced as early as 8 h after the treatment and further declined its steady level at 16 h with approximately 65% of the survived cells (Figure 11A). Dox caused dose-dependent cytotoxicity in HK-2 cells at low concentrations from 1 to 4 μM. For the higher dose, there was no further increase of dox-induced cytotoxicity in the cells was observed (Figure 11B).

2. Dox-induced apoptosis in human renal cells

Mode of the cell death observed above was then analyzed using Hoechst 33342 and propidium iodide (PI) stainings in order to detect whether it was from the mode of apoptosis or necrosis, respectively. Figure 12 shows that the DNA condensed cells stained by Hoechst 33342, the indicator of cell apoptosis, were clearly observed

while PI-positive necrosis cells were barely detectable. The results indicate that apoptosis is the major mechanism of renal cell damage caused by Dox. The apoptotic action was observed in a time-dependent manner as presented in Figures 12A and C. This Dox-induced apoptosis was initially detected at 12 h and gradually increased until 24 h. In term of dose-dependent study, Dox at 1-8 μM caused a significant decrease in the HK-2 cells survival (Figure 11B) and apoptosis was observed as the main type of cell death (Figures 12B and D). By estimation, 37% of HK-2 apoptotic cells were found from the treatment of 4-8 μM Dox in 24 h.

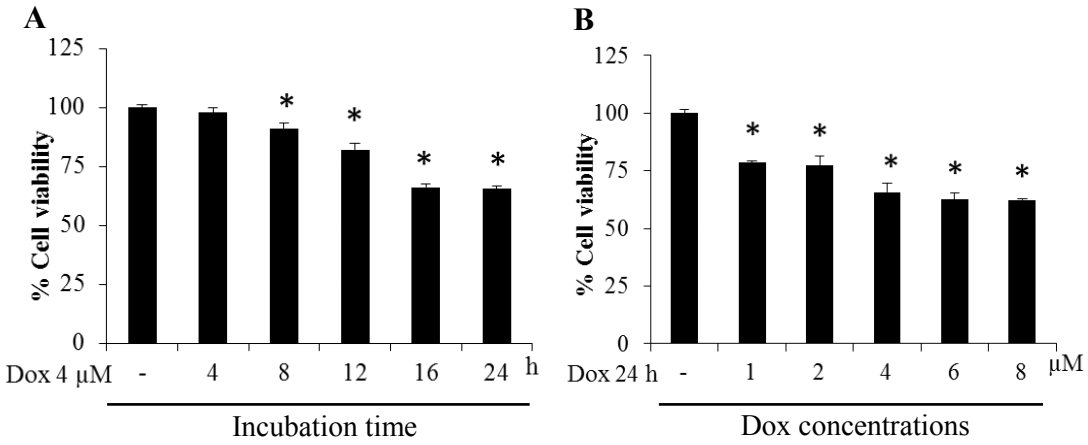


Figure 11. Cytotoxicity of doxorubicin in human renal cells. (**A**) Time-dependent toxicity profile generated by the treatment of HK-2 cells with 4 μM doxorubicin (Dox). (**B**) Dose-dependent toxicity of Dox (0-4 μM) after its 24 h treatment. MTT assay was used for the detection of viable cells. Cell viability values are means of triplicate samples±S.D. *, $p < 0.05$ versus non-treated control.

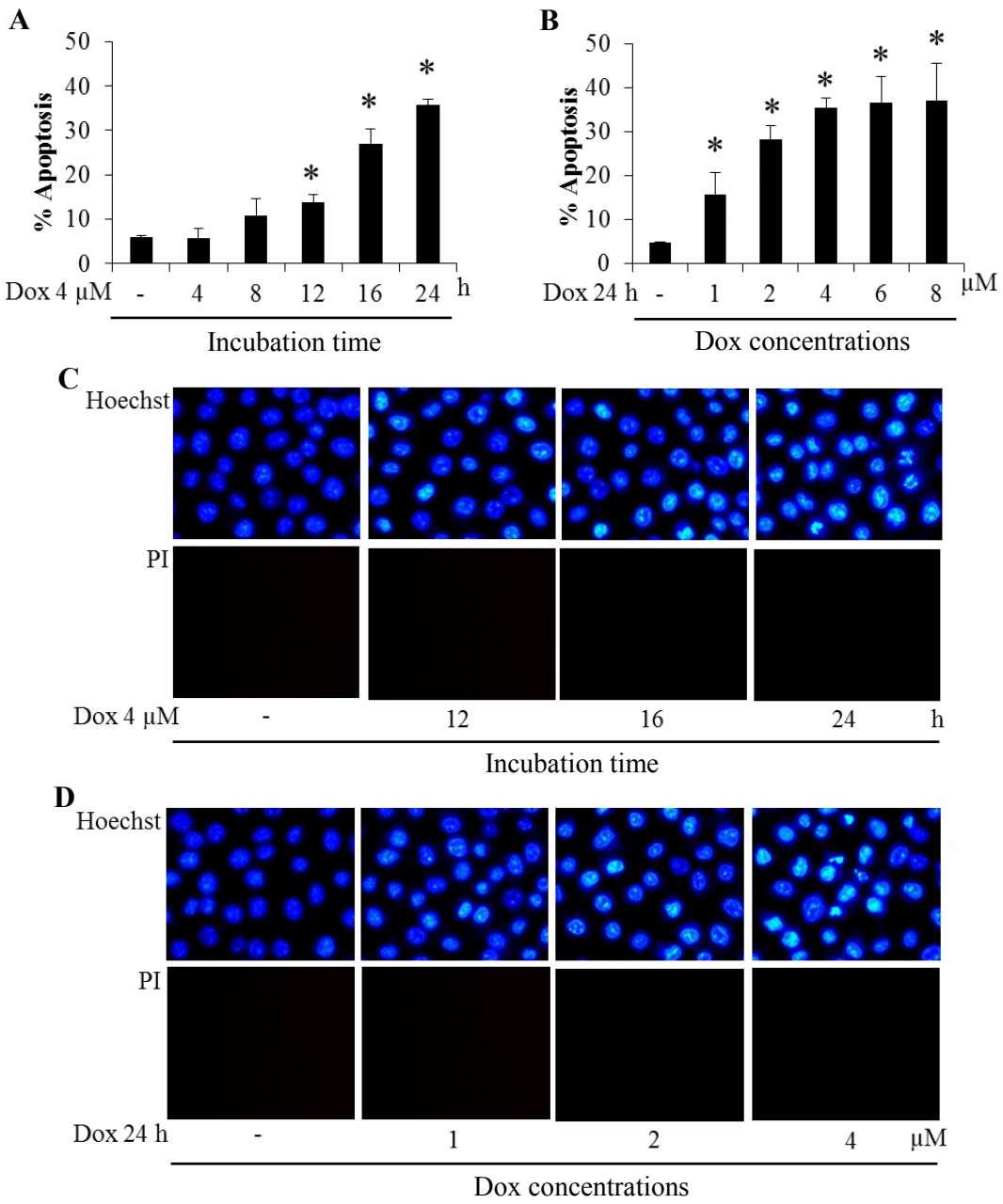


Figure 12. Hoechst 33342 and propidium iodide (PI) stainings showing apoptotic cell death caused by doxorubicin (Dox) in time- and dose-dependent manners. (A and C) DNA condensation was significantly detected after 12 h of Dox treatment. (B and D) The maximum percentage of the apoptosis was observed at 4-8 μM of Dox. The percentage of the values are means on triplicate samples±S.D. *, $p < 0.05$ versus non-treated control.

3. The decline of the anti-apoptotic protein, Mcl-1 in the Dox-induced renal toxicity

Among various cellular anti-apoptotic mechanisms, the protein functions of Bcl-2 family members have gained most attention. For Dox-mediated cell death, Mcl-1 has been shown to be closely related to sustain viability of cells in various cell types (Katoh et al., 1998; Lestini et al., 2009; Lopez-Royuela et al., 2010). Therefore, the effect of Dox on the cellular Mcl-1 and Bcl-2 level was investigated in the present study. As shown in Figures 13A and B, the significant reduction of Mcl-1 level was observed as early as 8 h after the Dox treatment at 4 μM. Furthermore, the western blot analysis of Mcl-1 and Bcl-2 proteins in the cells treated with various Dox concentrations (0-4 μM) indicated that the level of Mcl-1 was declined in a dose-dependent manner (Figures 13C and D). Nevertheless, the change of Bcl-2 level was not clearly observed in this study (Figure 13).

To demonstrate the vital role of Mcl-1 in the inhibition of Dox-mediated apoptosis in HK-2 cells, the cells were enforced to overexpress Mcl-1 protein by transient transfection of pcDNA3.1-Mcl-1 plasmid. After 36 h of the transfection, the Mcl-1 level was then determined by western blotting. The obtained results showed that the pcDNA-Mcl-1-transfected HK-2 cells had significant higher level of Mcl-1 compared to that of pcDNA3.1-tranfected HK-2 which is a control plasmid-transfected cells (Figures 14A and B). Cytotoxicity assay further revealed that the pcDNA-Mcl-1-transfection significantly rendered HK-2 cells resistant to Dox-mediated apoptosis (Figures 14D and E), supporting the role of Mcl-1 protein in the protection of Dox-mediated toxicity in renal cells.

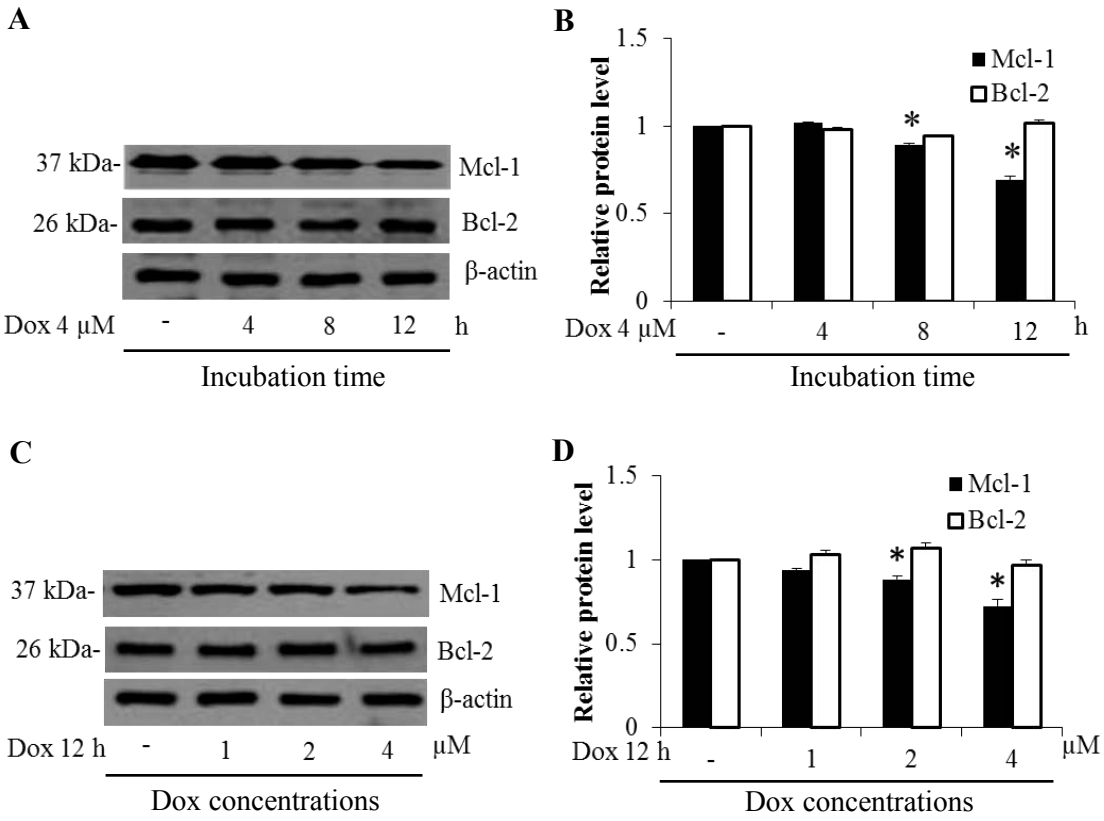


Figure 13. Western blot analysis showing the effect of doxorubicin on Mcl-1 level in human renal cells. (A and B) The reduction of Mcl-1 was significantly observed after treatment of doxorubicin (Dox) at 4 μM for 8 and 12 h. (C and D) Dose-dependent effect of Dox on the decline of Mcl-1 level but not Bcl-2 in HK-2 cells. Values are means on triplicate samples±S.D. *, $p < 0.05$ versus non-treated control.

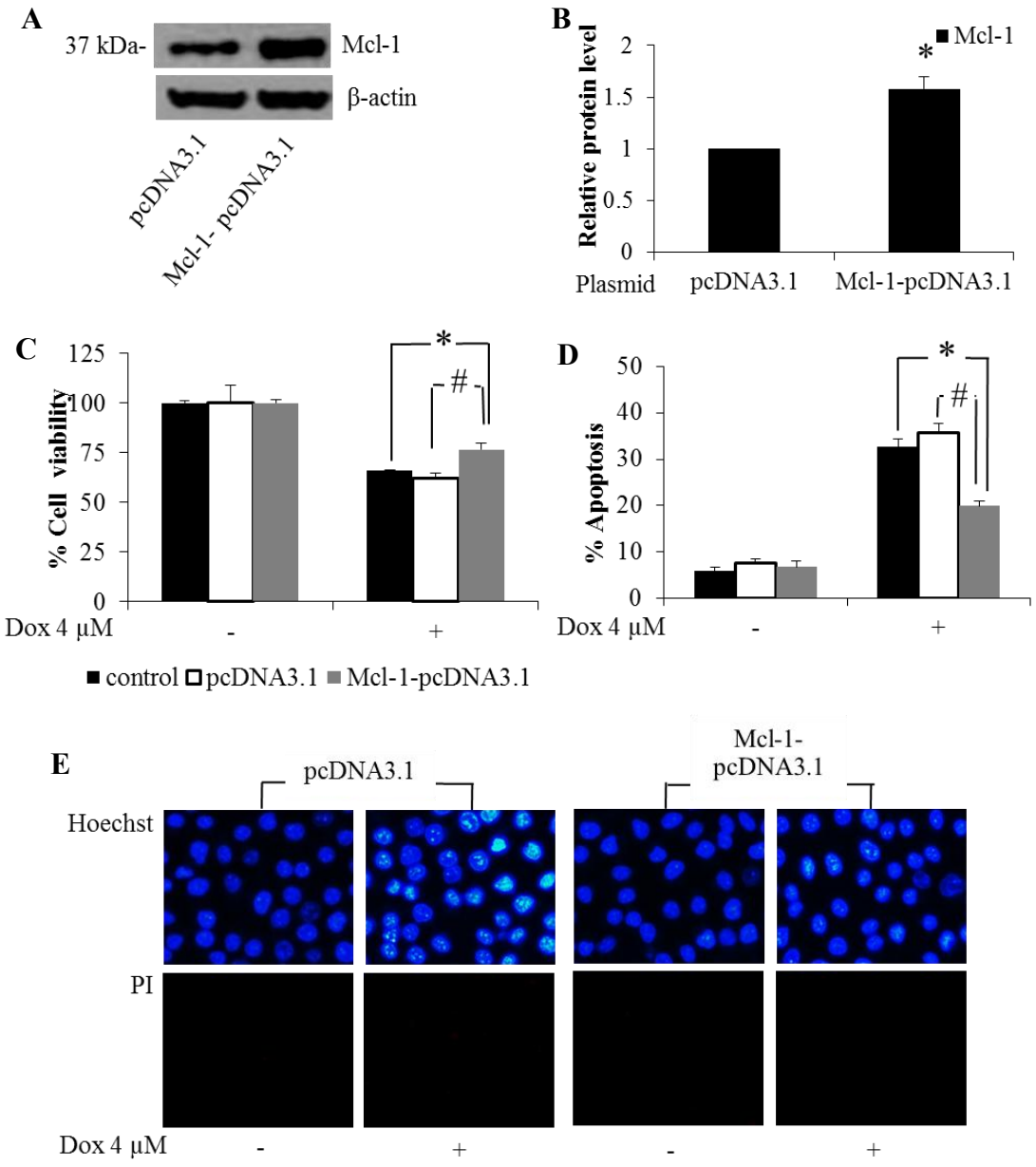


Figure 14. Overexpression of Mcl-1 in HK-2 cells preventing doxorubicin-induced apoptosis. (A and B) Western blotting showing the up-regulation of Mcl-1 in HK-2 cells through transfection of pcDNA-Mcl-1 plasmid. (C, D and E) The lower of %apoptotic cell was observed in the pcDNA-Mcl-1-transfected cells after doxorubicin (Dox) treatment. Values are means on triplicate samples±S.D. *, $p < 0.05$ versus non-transfected HK-2. #, $p < 0.05$ versus control plasmid-transfected HK-2 cells.

4. The preventive effect of plaunotol on Dox-induced apoptosis in human renal cells

In order to investigate the preventive effect of plaunotol on Dox-induced renal cell death, the sub-toxic concentrations of plaunotol in HK-2 cells was first clarified. After incubating with plaunotol at the concentrations of 0-100 μM for 24 h, cell viability was determined using MTT assay as previously described. The results showed that no significant difference of cell viability between non-treated cells and the cells treated with plaunotol ranging from 0-40 μM. However, with the higher concentration from 60 to 100 μM of plaunotol, the reduction of the cell viability could be detected with approximately 20% (Appendix A). Therefore, the protective effect of plaunotol was investigated by pretreated HK-2 cells with plaunotol at the lower concentrations of 0-40 μM for various times (0-12 h) prior the treatment of Dox at 4 μM. The results of cell viability and apoptosis after 24 h were determined by the methods of MTT and co-staining of Hoechst 33342 and propidium iodide, respectively. Interestingly, the protective effect of plaunotol against Dox-induced cell death was observed only when plaunotol pretreated the cells for at least 9 h. As shown in Figure 15, the pretreatment of plaunotol to the cells for 9 and 12 h could obviously prevent the apoptosis induced by Dox in HK-2 cells. With different concentrations of plaunotol, the pretreatment for 9 h also showed the preventive activity of plaunotol against Dox-induced renal cell death in a dose-dependent manner (Figure 16). These results suggest that plaunotol with appropriate concentration and pretreatment time can exert anti-apoptotic activity against Dox-induced toxicity in HK-2 cells. The results also imply that the alteration by plaunotol in the defensive mechanisms against cell death is a time requiring process.

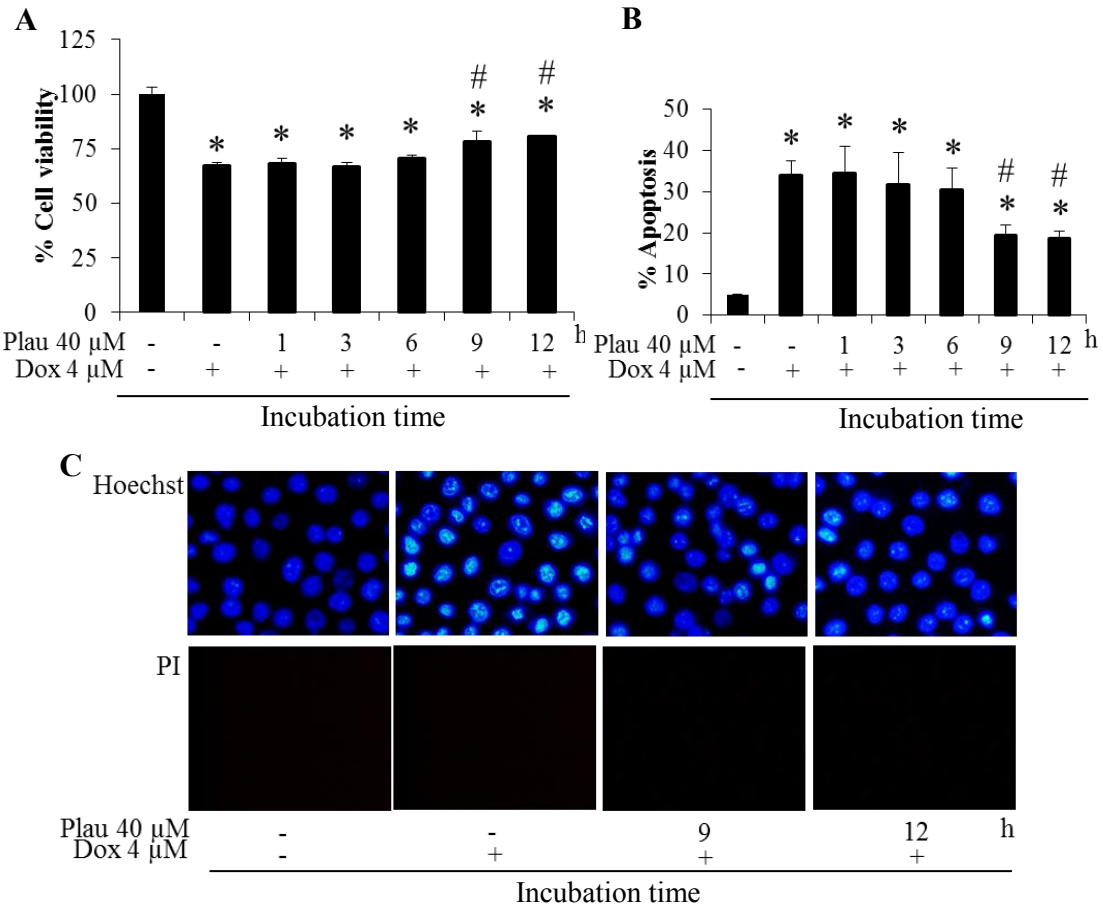


Figure 15. Protective effect of plaunotol on doxorubicin-induced human renal cell death. HK-2 cells were pretreated with 40 μM of plaunotol (Plau) at different time points (0-12 h) prior the treatment of doxorubicin (Dox) at 4 μM for 24 h. (A) Cell viability was measured by MTT assay. (B and C) The reduction of Dox-induced apoptosis was demonstrated in the pretreatment with plaunotol for 9-12 h. The representative photographs are shown from three independent experiments. Values are means on triplicate samples±S.D. *, $p < 0.05$ versus non-treated control. #, $p < 0.05$ versus Dox-treated control.

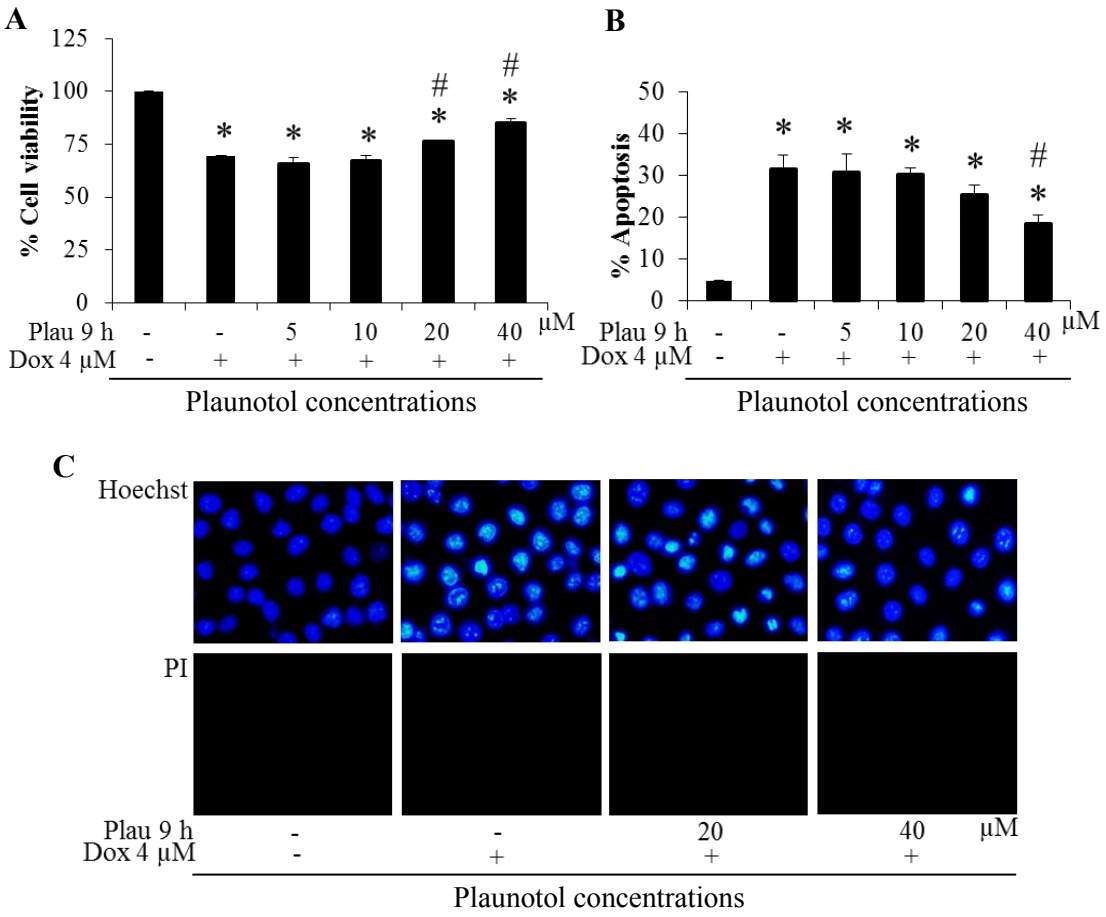


Figure 16. Dose-dependent effect of plaunotol in the prevention on doxorubicin-induced apoptosis in human renal cells. (A) Preincubation of HK-2 cells with 20 and 40 μM plaunotol (Plau) for 9 h prior doxorubicin (Dox) treatment render cell viability from cytotoxicity of Dox. (B and C) Hoechst 33342 and propidium iodide (PI) staining indicated dose-dependent lowering of apoptotic cell death in HK-2 cells pretreated with various concentration (0-40 μM) of plaunotol but only 40 μM of plaunotol significantly inhibited apoptosis induced by Dox. The representative photographs are shown from three independent experiments. Values are means on triplicate samples±S.D. *, $p < 0.05$ versus non-treated control. #, $p < 0.05$ versus Dox-treated.

5. High expression of Mcl-1 induced by plaunotol in human renal cells

From Figure 14, the overexpression of Mcl-1 in human renal cells was observed to be a possible cause of protection against Dox-induced toxicity. Thus the expression of Mcl-1 as a result of plaunotol protective effect was further investigated. Human renal HK-2 cells were incubated with plaunotol at the concentrations of 0-40 μM for 0-9 h. The expression of Mcl-1 was then evaluated by western blotting with anti-Mcl-1 antibody. The results are shown in Figures 17A and B. It was found that at the dose of 40 μM, the treatment of plaunotol resulted in significant up-regulation of Mcl-1 level at 6 h and 9 h compared to non-treated control. It should be noted that even the level of Mcl-1 in response to plaunotol treatment at 6 h was also significantly increased, but the protein level might not be sufficient to protect cell death induced by Dox. Also, the dose-dependent expression of MCl-1 level induced by plaunotol (Figures 16C and D) showed its well correlation with the protective effect of plaunotol at the concentrations 20-40 μM.

To substantiate these findings, the effect of Dox on Mcl-1 level in HK-2 cells in the presence and absence of plaunotol was further examined. Cells were left untreated or pretreated with 10-40 μM plaunotol for 9 h and incubated with toxic concentration of Dox for 12 h. Western blot analysis for the Mcl-1 level was then performed. It was found that Dox treatment reduced the cellular Mcl-1 level in a dose-dependent manner (Figure 18) which was well consistent with its toxic action presented in Figures 12B and D. It was also found that the Mcl-1 up-regulation induced by plaunotol pretreatment was able to overcome the reductive effect of Dox and rendered the survival of the renal cells in this condition.

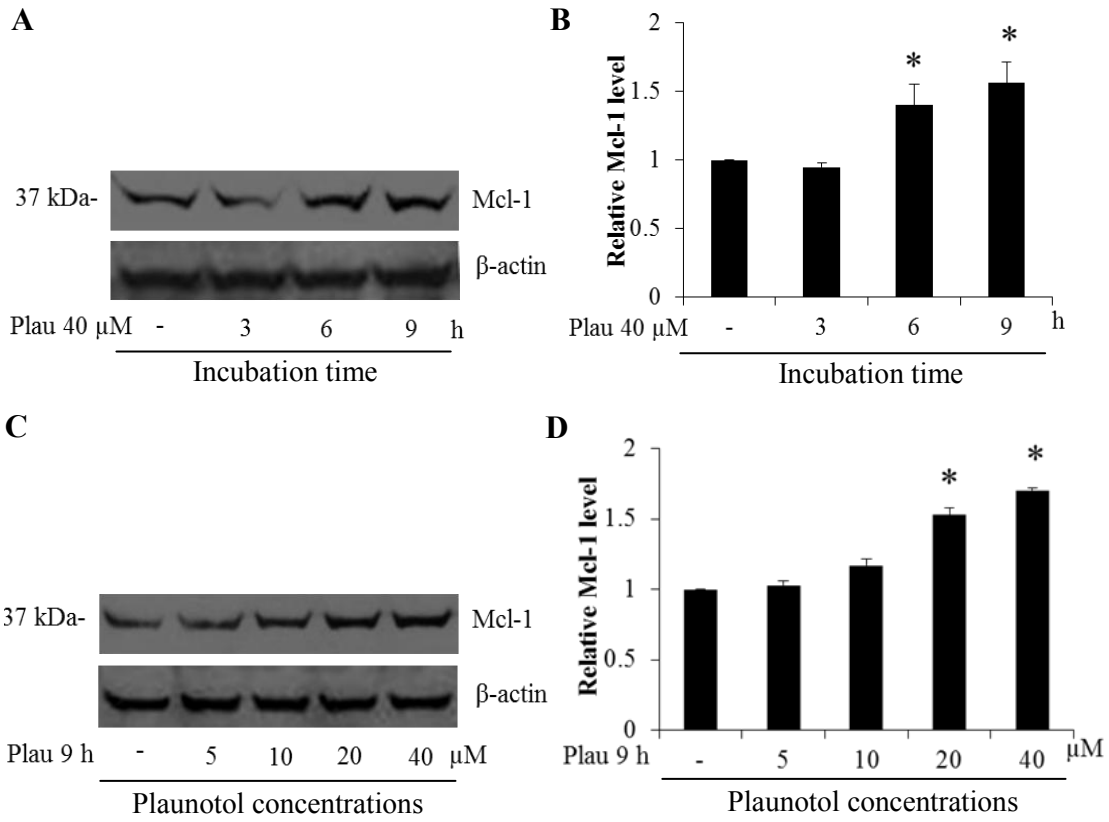


Figure 17. The effect of plaunotol on the anti-apoptotic protein Mcl-1. (A and B) The expression of Mcl-1 were examined by western blotting after incubation of HK-2 cells with 40 μM plaunotol (Plau) for 0-9 h. (C and D) Dose-dependent effect of plaunotol (0-40 μM) on up-regulation of Mcl-1 level in HK-2 cells. Values are means on triplicate samples±S.D. *, $p < 0.05$ versus non-treated control.

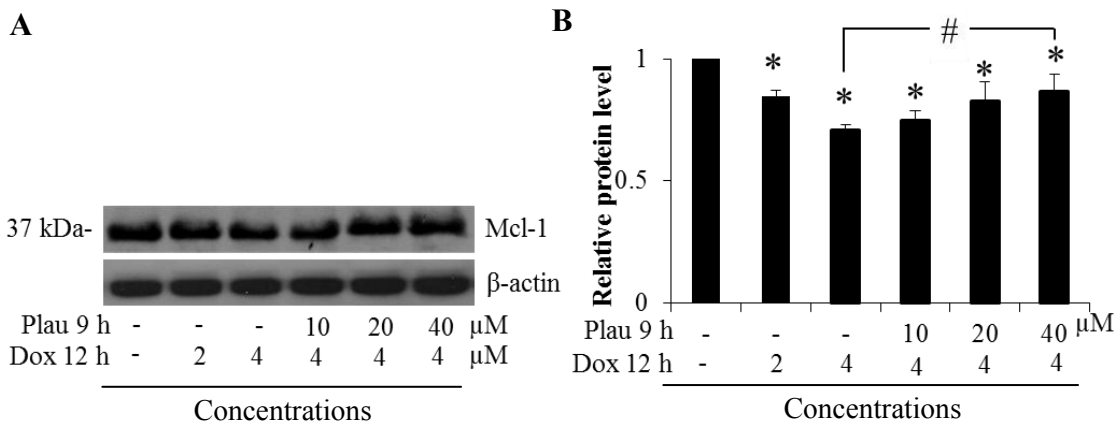


Figure 18. Recovery effect of plaunotol on Mcl-1 down-regulation induced by doxorubicin in human renal cells. (A and B) The cells were pretreated with plaunotol (Plau) for 9 h before being treated with 4 μM Dox for 12 h. Western blotting ws then performed to investigate Mcl-1 level. Values are means on triplicate samples±S.D. *, $p < 0.05$ versus non-treated control, and #, $p < 0.05$ versus Dox-treated control.

6. Inhibitory effect of plaunotol on Mcl-1 down-regulation induced by oxidative stress

As reported in various studies, reactive oxygen species (ROS) have been proved to regulate the degradation of Mcl-1 (Chiu et al., 2012; Inoshita et al., 2002; Jang et al., 2010; Kang et al., 2008). In this case, therefore, Mcl-1 down-regulation induced by ROS, and the underlying mechanism of plaunotol in the up-regulation of Mcl-1 were investigated in human renal cells. DMNQ was chosen to generate oxidative stress because its capability to produce superoxide anion ($O_2^{\cdot-}$), a precursor ROS, in order to mimic the ROS production at normal cell condition. Practically, the fluorescent intensity of dihydroethidium (DHE), a specific probe for $O_2^{\cdot-}$, in human

renal HK-2 cells was evaluated under fluorescent microscope and microplate reader after 2 h of incubation with 10 μM DMNQ.

Successful induction of oxidative stress was indicated by the higher DHE intensity in the DMNQ-treated cells. However, pretreatment with 50 μM MnTBAP, a specific scavenger for $O_2^{\cdot-}$, could prevent the increase of $O_2^{\cdot-}$ in HK-2 cells (Figures 19A and B). Interestingly, western blotting obviously showed the alteration of Mcl-1 level corresponding to DHE fluorescent intensity (Figures 19C and D). Treatment of DMNQ not only generated ROS but also significantly reduced Mcl-1 level in renal HK-2 cells. Down-regulation of Mcl-1 was rendered by pretreatment of MnTBAP for 1 h prior DMNQ treatment. These results confirm with previous studies which indicated that the regulation of Mcl-1 level depends on the cellular oxidative stress, and antioxidant substance sufficiently restores the reduction Mcl-1 induced by ROS.

Base on the results, further investigated the effect of plaunotol in prevention on Mcl-1 down-regulation in the cell treated DMNQ. DMNQ-induced oxidative stress in HK-2 cells could be inhibited by pretreatment of 40 μM plaunotol for 3 h (Figures 19A and B). Surprisingly, this pre-incubation of plaunotol significantly prevented the reduction of Mcl-1 in HK-2 treated with DMNQ. Noteworthy, treatment with 40 μM plaunotol for 3 h did not alter the level of Mcl-1 in human renal cells (Figures 17A and B). Together, these results demonstrate that plaunotol presents antioxidant activity in human renal cells and manipulates the expression of Mcl-1 through ROS scavenging activity. Thus, the antioxidant capacity of plaunotol in human renal cells was further investigated.

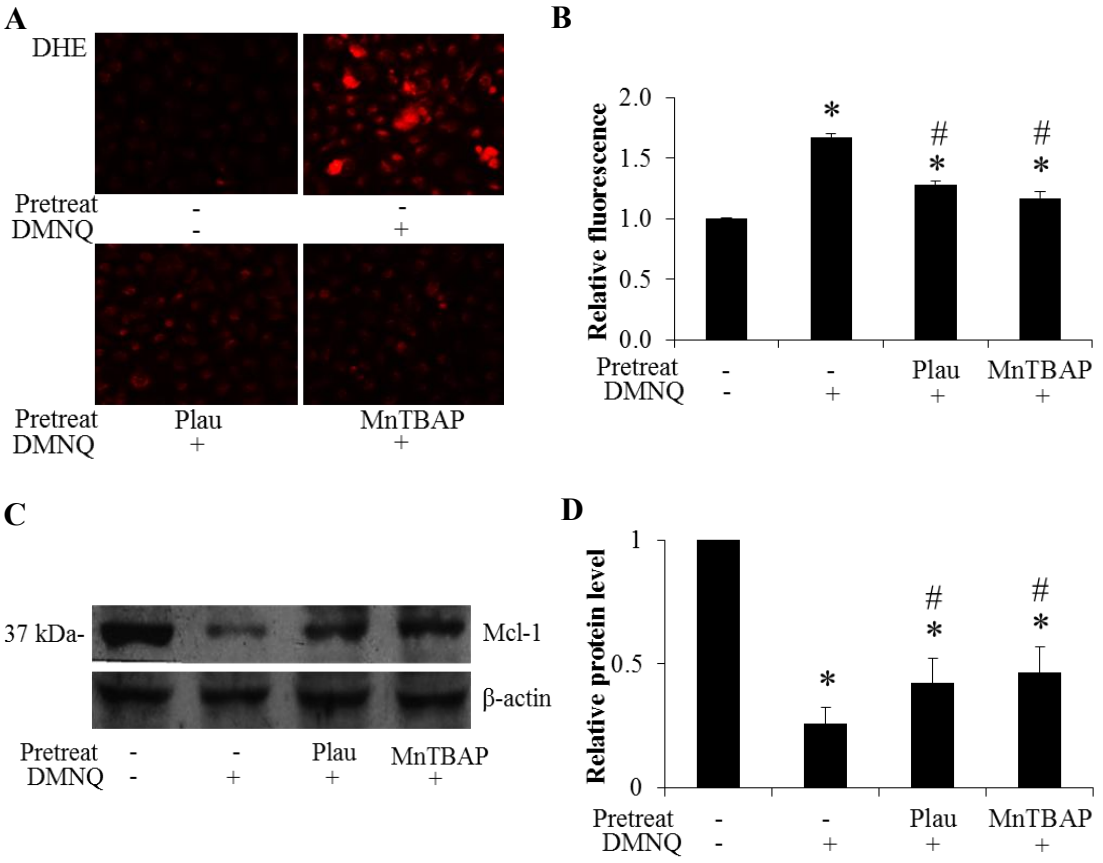


Figure 19. The effect of plaunotol on the Mcl-1 down-regulation induced by ROS. The generation of $O_2^{\cdot -}$ in HK-2 cells was presented as indication with the increase of DHE fluorescent intensity after treatment of 10 μM DMNQ for 2 h. Cells were pretreated with 50 μM MnTBAP for 1 h or 40 μM plaunotol (Plau) for 3 h prior treatment of DMNQ. (A and B) The reduction of DHE intensity was observed in HK-2 cells pretreated with MnTBAP and plaunotol via fluorescent microscope and microplate reader. (C and D) Western blot analysis showed the down-regulation of Mcl-1 after treatment with DMNQ, and the restorations of Mcl-1 in HK-2 cells pretreated of MnTBAP or plaunotol. The representative photographs are shown from three independent experiments. Values are means on triplicate samples±S.D. *, $p < 0.05$ versus non-treated control. #, $p < 0.05$ versus DMNQ-treated control.

7. Effect of plaunotol on the endogenous ROS in human renal cells

In order to characterize the antioxidant activity of pluanotol in human renal cells, its capability to decrease the basal cellular ROS level was first investigated. After incubation with plaunotol, the ROS level in human renal cells was detected by a ROS fluorescent probe, 2',7'-Dihydrodichlorofluorescein diacetate (H_2DCF-DA). As demonstrated in Figure 20, the decrease of DCF fluorescent intensity was observed in the HK-2 cells incubated with well-known antioxidants, N-acetylcysteine (NAC) or glutathione (GSH) for 1 h. Treatment of the cells with 40 μM plaunotol for at least 3 h significantly reduced the basal ROS level (Figure 20A).

Noteworthy, the increase of Mcl-1 level in human renal cells treated with plaunotol for 6-12 h (Figures 17A and B) was sequentially observed after its antioxidant activity against basal cellular ROS. These data indicates that plaunotol up-regulates Mcl-1 level in human renal cells via its antioxidant activity. Dose-dependence of antioxidant activity was also found in the cells treated with plaunotol at various concentrations. The significant lowing of endogenous ROS was presented in 20 and 40 μM of plaunotol treatment (Figure 20B).

8. Specific ROS scavenging activity of plaunotol in human renal cells

The specific antioxidant activity of plaunotol against three hazard ROS, including superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot}$) in human renal cells was further clarified.

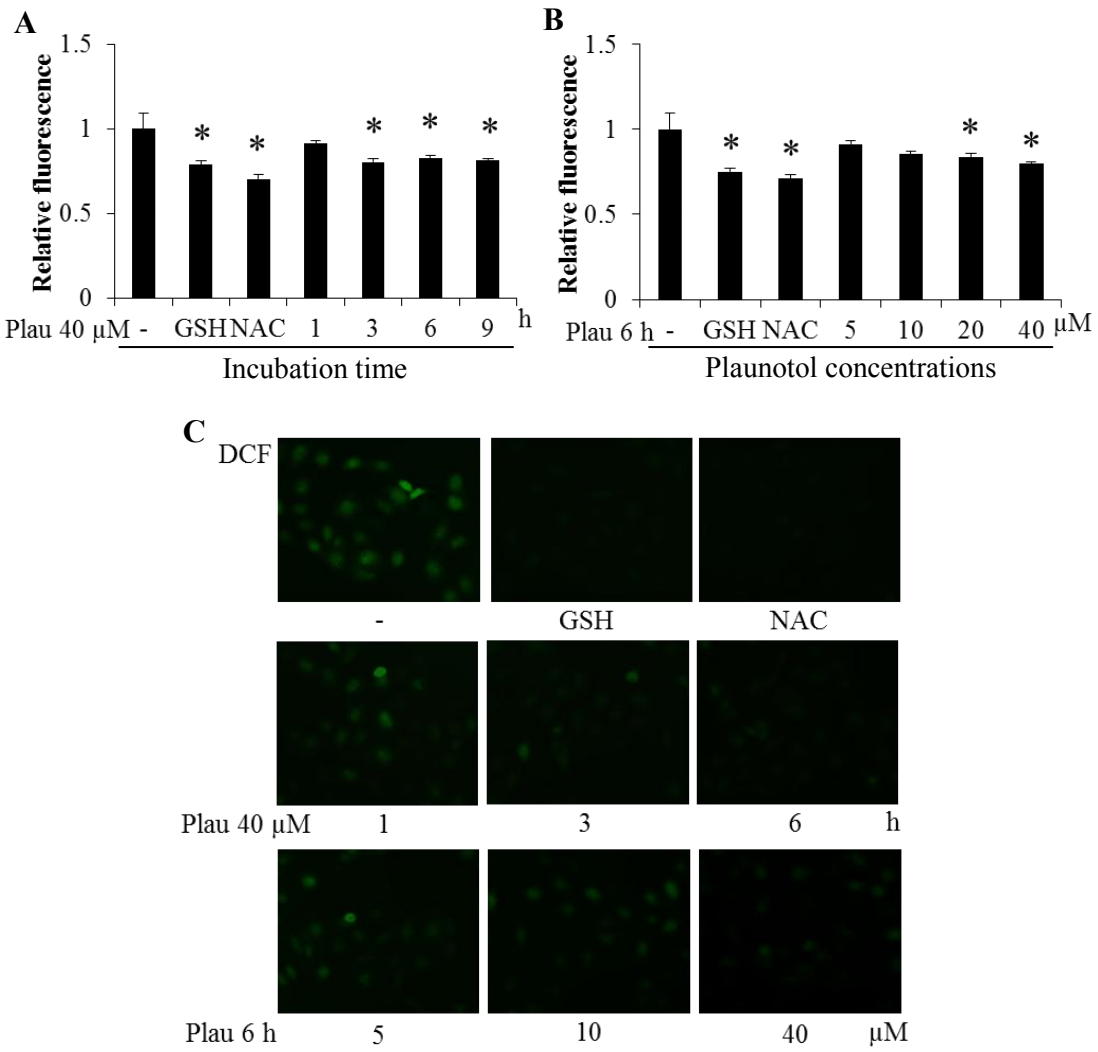


Figure 20. The effect of plaunotol on the demolishment of endogenous ROS in human renal cells. (A) Significant reduction of basal ROS level (as indicated by the lower DCF intensity) in HK-2 cells treated with 1 mM of NAC or GSH for 1 h as well as treatment of 40 μM plaunotol (Plau) for 3-9 h. (B) Dose-dependent manner of the antioxidant activity of plaunotol after incubation for 6 h. (C) The lower fluorescence of DCF in the cells treated with plaunotol was significantly observed under the fluorescent microscope. The representative photographs are shown from three independent experiments. Values are means on triplicate samples±S.D. *, $p < 0.05$ versus non-treated control.

The cells were pretreated with plaunotol at various concentrations (0-40 μM) and for various time intervals (0-6 h) prior to the treatment of specific ROS generators**:** DMNQ, H_2O_2 and combination of ferrous ion (Fe^{2+}) and H_2O_2. Superoxide anion ($O_2^{\cdot-}$) was significantly observed via the increase of DHE fluorescent intensity after 2 h of incubation with DMNQ in HK-2 cells (Figure 21). The demolishment of $O_2^{\cdot-}$ was found in the cells pretreated with NAC, GSH and plaunotol. Similar to the previous results, the antioxidant activity of plaunotol against $O_2^{\cdot-}$ was observed in the cells pretreated with 40 μM plaunotol for at least 3 h. The increase of DCF fluorescent intensity about 2.5 fold indicated that the level of H_2O_2 was significantly presented after 30 min of the adding 100 μM H_2O_2 into the cells (Figure 22). Pre-incubation of HK-2 cells with 40 μM plaunotol resulted in the decrease of H_2O_2 in time-dependent manner (Figure21 A). The dose dependence of the antioxidant activity against H_2O_2 was also observed in HK-2 cells pretreated with plaunotol (Figure21 B).

According to Fenton reaction, hydroxyl radical ($OH^{\cdot}$) has been generated in human renal cell through the combination treatment of Fe^{2+} and H_2O_2 (Nordberg and Arner, 2001; Valko et al., 2007). In this study, after the treatment with ferrous sulfate following with 100 μM H_2O_2 for 30 min, the intensity of hydroxyphenyl fluorescein (HPF), a specific probe for $OH^{\cdot}$, was determined under fluorescent microscope and microplate reader. The increase of $OH^{\cdot}$ was demonstrated in Figure 23. NAC and GSH significantly scavenged $OH^{\cdot}$ as indicated with the reduction of HPF fluorescent intensity. Although plaunotol could demolish $OH^{\cdot}$, it required a longer pretreatment time for at least 6 h to exert the specific antioxidant activity against $OH^{\cdot}$.

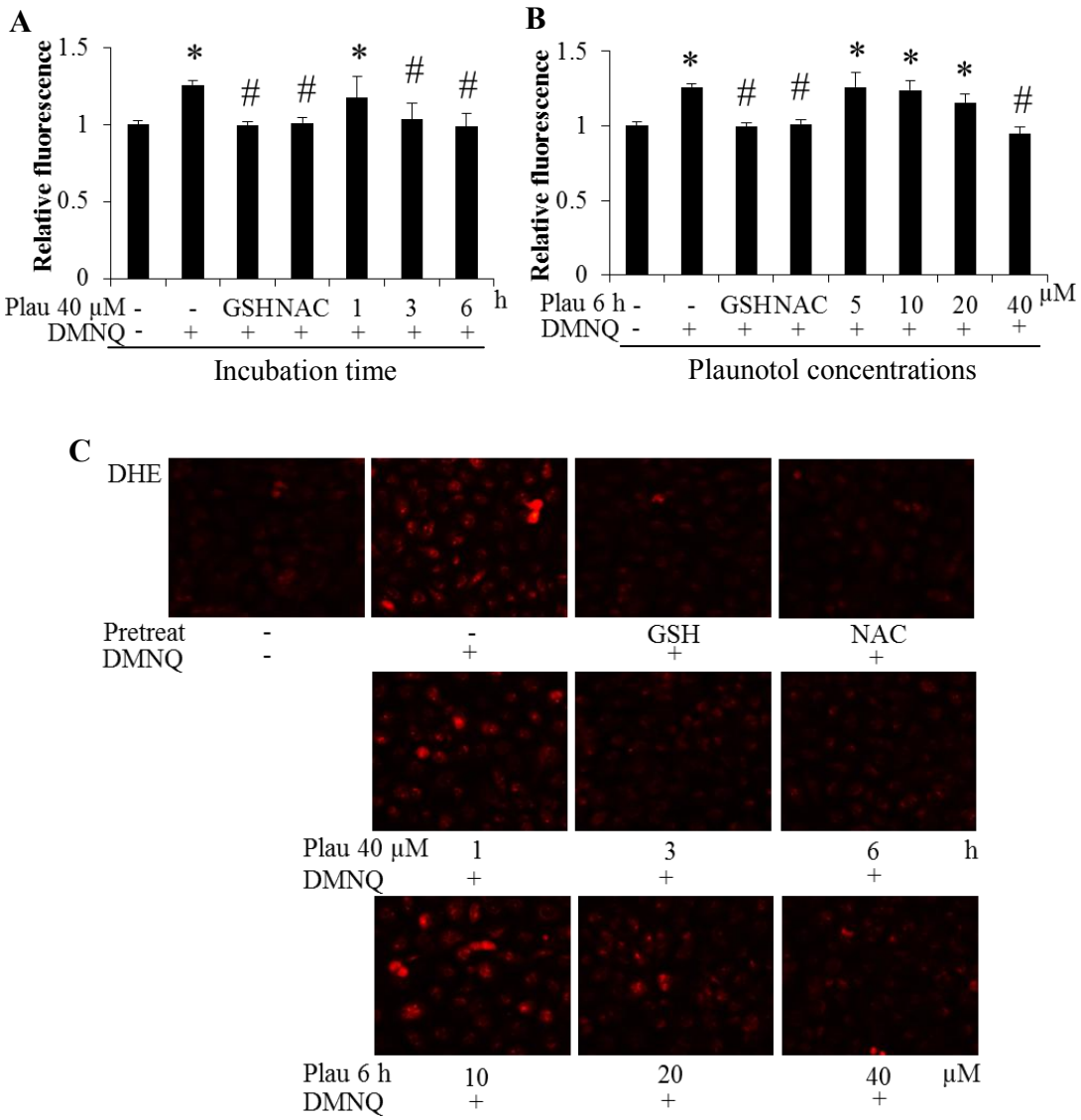


Figure 21. Antioxidant activity of plaunotol against superoxide anion in human renal cells. (A and B) After treatment with 10 μM DMNQ for 2 h, superoxide anion ($O_2^{\cdot-}$) in HK-2 cells was detected by the fluorescent intensity of DHE via fluorescent microplate reader. (C) The reduction of $O_2^{\cdot-}$ in the cells pretreated with plaunotol (Plau) was also visualized under a fluorescent microscope. The representative photographs are shown from three independent experiments. Values are means on triplicate samples±S.D. *, $p < 0.05$ versus non-treated control. #, $p < 0.05$ versus DMNQ-treated control.

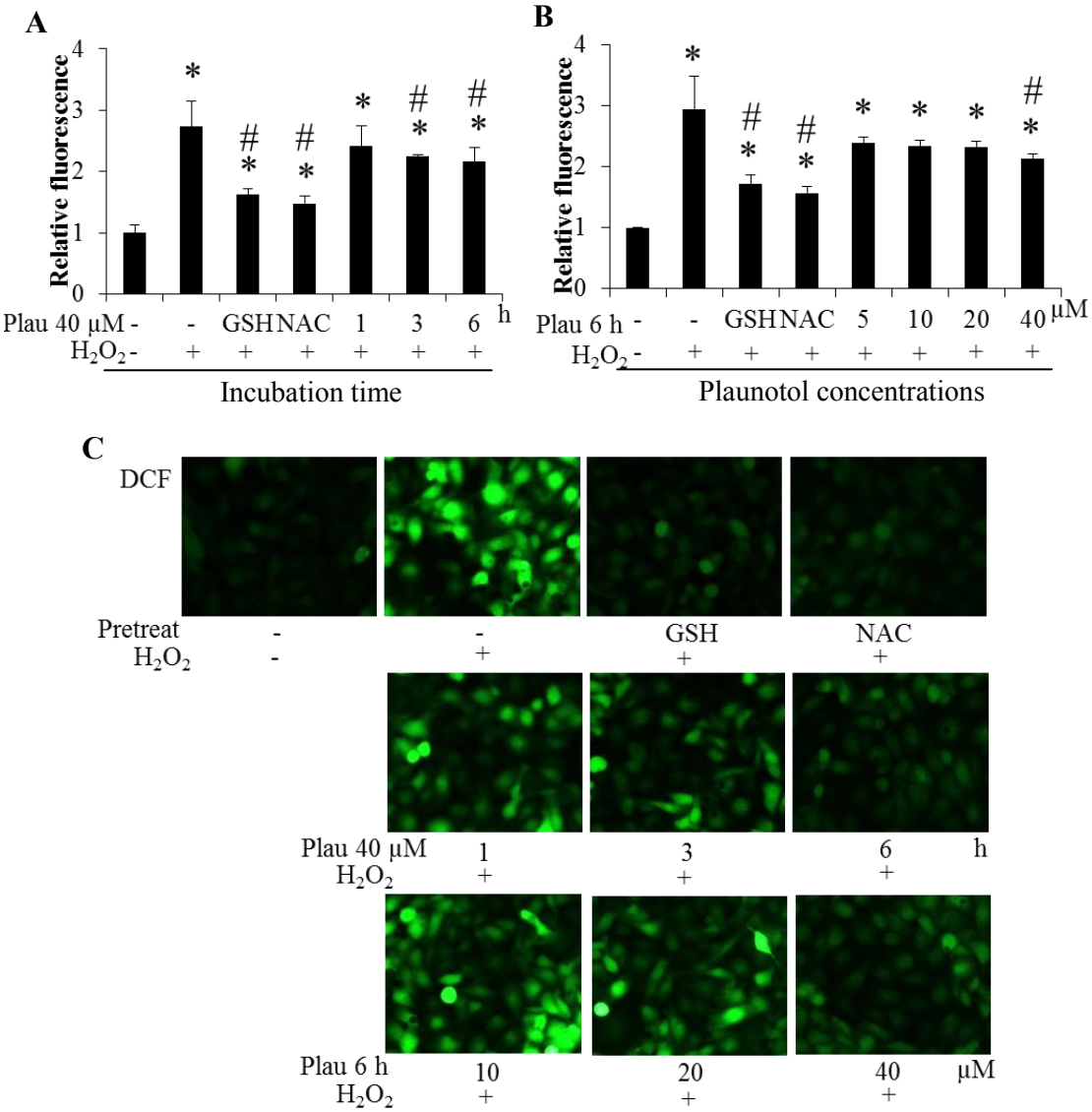


Figure 22. Antioxidant activity of plaunotol against hydrogen peroxide in human renal cells. (A and B) Relative fluorescent intensity of DCF showed the lower level of hydrogen peroxide (H_2O_2) in HK-2 cells pretreated with NAC, GSH and plaunotol (Plau) prior to treatment of 100 µM of H_2O_2. (C) The DCF fluorescence in the cells was examined under a fluorescent microscope. Representative photographs are shown from three independent experiments. Values are means on triplicate samples±S.D. *, $p < 0.05$ versus non-treated control. #, $p < 0.05$ versus H_2O_2-treated control.

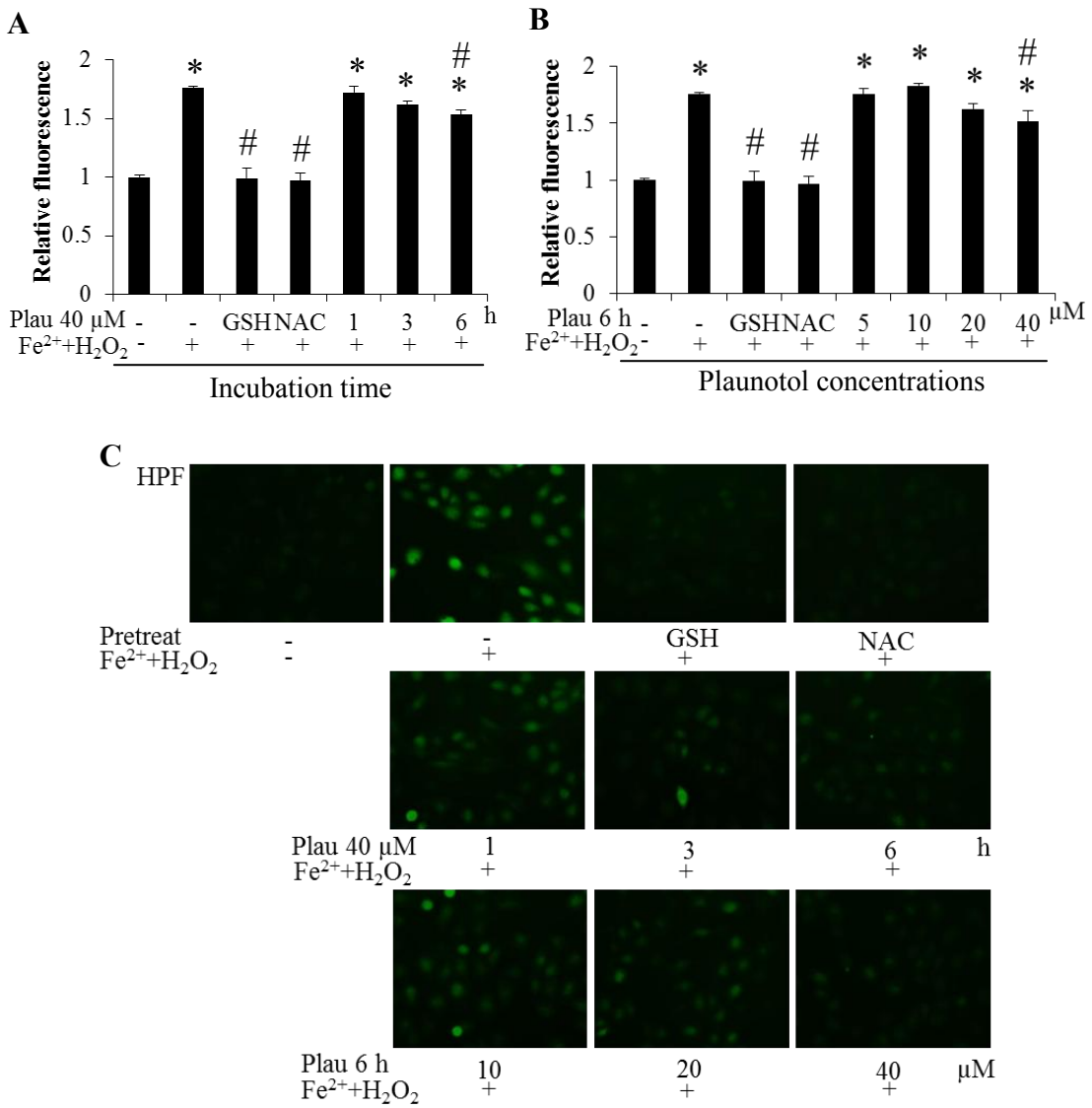


Figure 23. Antioxidant activity of plaunotol against hydroxyl radical in human renal cells. (A and B) Hydroxyl radical ($OH^{\cdot}$) in HK-2 cells was generated by treatment of Fe^{2+}+H_2O_2 for 30 min. The fluorescent intensity of HPF was examined under a fluorescent microplate reader. (C) Time and dose-dependent antioxidant activity of plaunotol (Plau) against $OH^{\cdot}$ was indicated via visualization under fluorescent microscope. Representative photographs are shown from three independent experiments. Values are means on triplicate samples±S.D. *, $p < 0.05$ versus non-treated control. #, $p < 0.05$ versus Fe^{2+}+H_2O_2-treated control.

9. The effect of plaunotol on the glutathione content in human renal cells

The antioxidant mechanism of plaunotol was further investigated in both non-cell based and cell based assay. The ROS scavenging activity of plaunotol was examined by DPPH and ORAC assay. As demonstrated in Table1, the well-known antioxidants, N-acetylcysteine (NAC) and glutathione (GSH) exhibited the capability to interact with free radical ($DPPH^{\cdot}$) indicating with high percentage of scavenging activity in DPPH assay. The scavenging capability against $ROO^{\cdot}$ of NAC and GSH was also presented in ORAC assay as Trolox equivalent value (μmole of Trolox per 1 mg of substance). However, the scavenging activity of plaunotol was barely detected from both DPPH and ORAC assay.

Table 1. Non-cell based assay for determining the antioxidant activity of plaunotol

	DPPH assay % Scavenging activity	ORAC assay Trolox equivalents (μmole/mg)
GSH	39.40±1.13	1.48±0.11
NAC	60.31±0.51	5.60±0.54
Plaunotol	0.17±0.29	0.57±0.37

The effect of plaunotol on cellular antioxidant mechanism in human renal cells was also investigated. Total glutathione (GSH) content in HK-2 cells which were treated with plaunotol was detected by monochlorobimane (MCB) reaction. After 6 h of incubating the cells with 5 μM NAC, a substrate for GSH synthesis, there was a significant increase of cellular GSH in HK-2 cells.

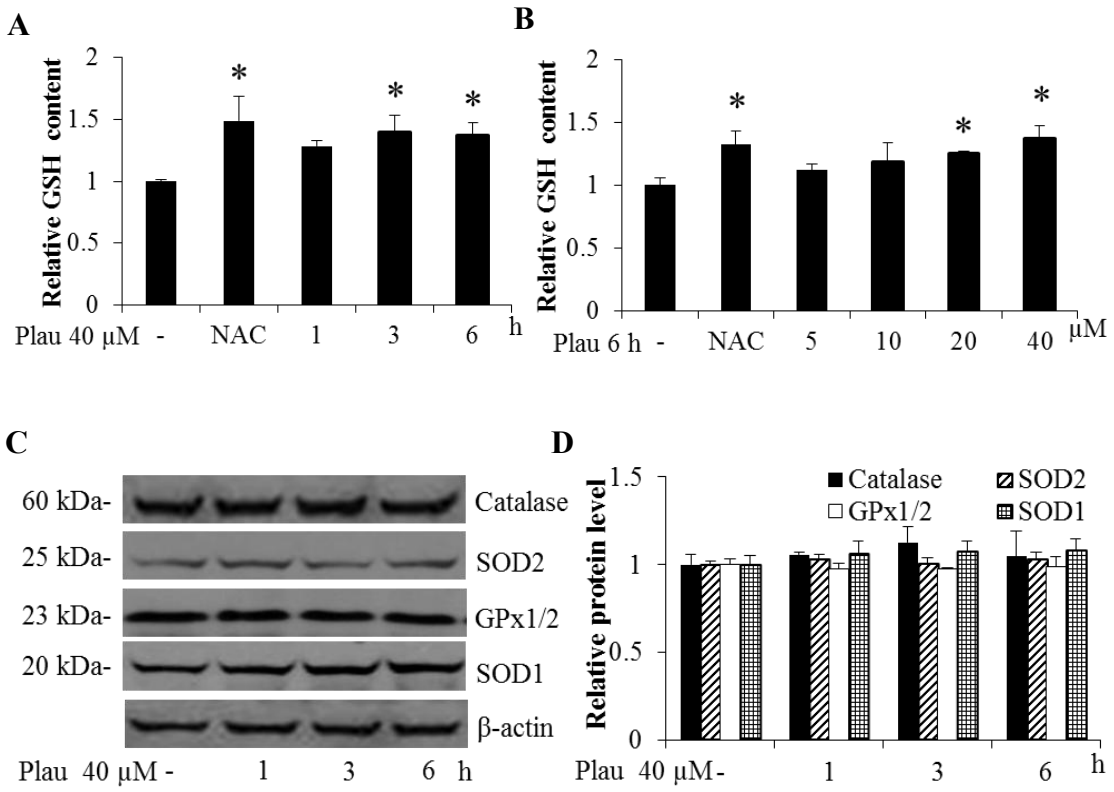


Figure 24. The effect of plaunotol on cellular antioxidant mechanism in human renal cells. (A and B) The increase of relative glutathione (GSH) content in HK-2 cells after incubation of the cells with NAC and plaunotol (Plau) was detected via monochlorobimane (MCB), and calculated compared with non-treated control cells. (C and D) Western blot analysis revealed the level of antioxidant enzymes in HK-2 cells after treatment with 40 µM plaunotol for 1-6 h. Values are means on triplicate samples±S.D. *, $p < 0.05$ versus non-treated control.

The augmentation of GSH was also presented in HK-2 cells treated with plaunotol. The time- and dose-dependent effect of plaunotol on the induction of cellular GSH was indicated in Figures 24A and B. The significant increase of GSH

level after being treated with 40 μM plaunotol for 3 h well correlated with its antioxidant activity in human renal cells (Figures 20-23). Moreover, the alteration of enzymatic antioxidant induced by plaunotol was also examined. Figures 24C and D, the protein levels of vital antioxidant enzymes including manganese-superoxide dismutase (Mn-SOD), cupper/zinc-superoxide dismutase (Cu/Zn-SOD), catalase, and glutathione peroxidase (GPx) were found to be present. Western blotting revealed non-alteration of these antioxidant proteins in HK-2 cells after incubation with plaunotol. These results suggeste that the antioxidant capability of plaunotol in human renal cells does not present through direct ROS scavenging but come from the induction of cellular GSH content.

10. Selectivity of protective effect of plaunotol against Dox-induced cancer cell damage

Having shown the protective potential of plaunotol in Dox-mediated renal cell damage, the investigation on whether such Mcl-1 potentiating effect succor chemotherapeutic resistance in cancers was further clarified. In fact, the increase of Mcl-1 level is an important mechanism of chemotherapeutic resistance in various cancer cells including non-small cell lung cancer and melanoma (Chetoui et al., 2008; Wang et al., 2008; Khodadoust et al., 2009; Chen et al., 2010). Human non-small lung cancer (H460) and human melanoma (G361) were left untreated or pretreated with 40 μM plaunotol for 9 h and followed by the treatment of 4 μM Dox. After 24 h, cell viability and apoptosis, were determined. In cancer cells, treatment with 4 μM of

Dox alone caused cell survival reduction by approximately 20% in H460 and G361 cells (Figures 25A and 26A).

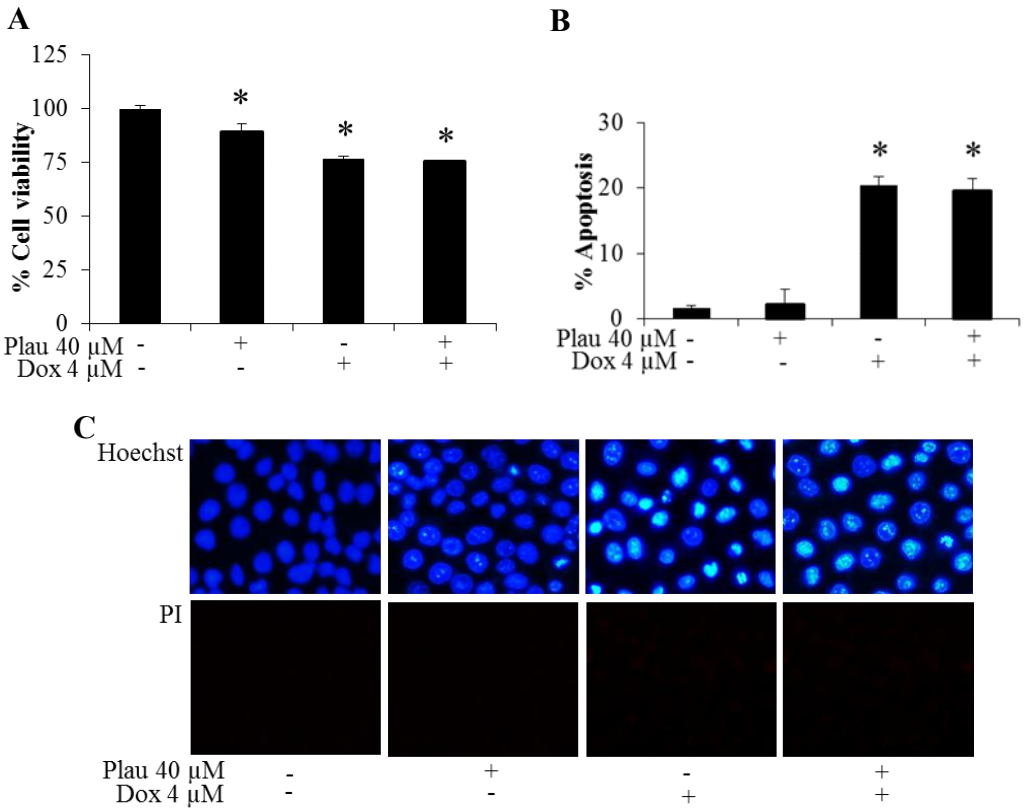


Figure 25. Effect of plaunotol on doxorubicin-induced toxicity in human non-small cell lung carcinoma. (A) H460 cells was either pretreated with 40 µM plaunotol (Plau) for 9 h or left untreated prior to treatment of 4 µM doxorubicin (Dox) for 24 h. MTT assay was then performed to indicate viability of the cells. (B and C) Hoechst 33342 staining showed that pretreatment of plaunotol did not alter Dox-induced apoptosis in H460 cells. The representative photographs are shown from three independent experiments. Values are means on triplicate samples±S.D. *, $p < 0.05$ versus non-treated control. #, $p < 0.05$ versus Dox-treated.

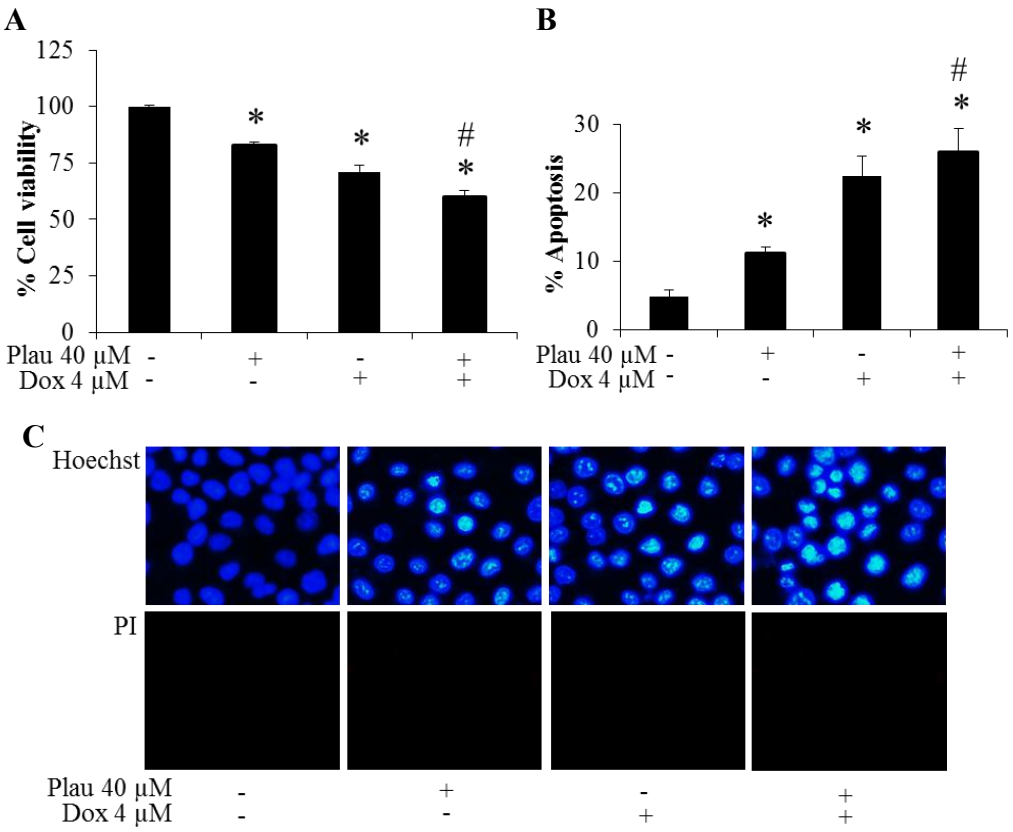


Figure 26. Sensitizing effect of plaunotol on doxorubicin-induced toxicity in human melanoma cells. (A) MTT analysis showed the significant lower of cell viability in human melanoma cells with pretreatment of 40 µM plaunotol (Plau) prior to treatment of 4 µM doxorubicin (Dox). (B and C) Pretreatment of G361 cells with plaunotol increased Dox-induced apoptosis as indicated with apoptotic body staining with Hoechst 33342. The representative photographs are shown from three independent experiments. Values are means on triplicate samples±S.D. *, $p < 0.05$ versus non-treated control. #, $p < 0.05$ versus Dox-treated.

Importantly, pretreatment of plaunotol in this experiment showed no alterative effect on apoptotic cell death induced by Dox in H460 as shown in Figures 25B and C. Further, plaunotol pretreatment exhibited sensitizing effect on Dox-induced apoptosis in melanoma cell G361 (Figures 26B and C). Even though the underlying mechanism of such selectivity is obscured these findings provide the initial evidence of plaunotol in selective protection against Dox-induced human renal cell damage.

CHAPTER V

DISCUSSION AND CONCLUSION

Renal injury is an incurrent disease that is often found in various pathobiologies such as diabetes, hypertension and chemotherapeutic condition (Bergamini and Seghieri, 2006; Ichikawa et al., 1994). The benefit of various chemotherapeutic medicines including doxorubicin has been limited by its severe side effect on renal damage (Burke et al., 1977). Doxorubicin decreases the renal function and causes several histopathological changes such as the damage of glomerular and tubular epithelial cells. The degeneration of renal proximal tubule cells has been observed in mice that are administered with doxorubicin (Cheng et al., 2006; Ueda and Shah, 2000; Zhang et al., 1996). In consistent, the present study found that treatment of doxorubicin caused cytotoxicity to human proximal tubular cells. Cell death analysis revealed that doxorubicin at the concentrations of 1–8 μM induced apoptosis in HK-2 cells in dose- and time-dependent manners (Figures 11 and 12).

Nowadays, increasing attention is paid to the chemopreventive therapy. Many substances have been proposed for their protective effects against doxorubicin-induced renal toxicity, and natural extracts have become the center of this interest because of their high potential in various therapeutic effects and human safety profile (Paduch et al., 2007). The extracts from various plants such as turmeric, rosemary and ginkgo have been demonstrated to preclude nephrotoxicity through their antioxidant activity (Abd-Ellah and Mariee, 2007; Mohamad et al., 2009; Sakr and Lamfon, 2010). However, the natural antioxidant possessing high protective efficiency with

minimal toxicity to other cells and none alteration on therapeutic activity of a current medicine remains under investigated. The present study aimed to test the protective capability of plaunotol, an acyclic diterpene from *C. stellatopilosus* Ohba (Plau-Noi) against doxorubicin toxicity in human renal cells. Plaunotol has been shown to prevent the degeneration of gastric epithelial cell from various stimuli (Murakami et al., 1999; Ohta et al., 2005). In addition, plaunotol is relatively non-toxic and has been approved for treatment of gastric and peptic ulcer treatment in human (Parejo et al., 2000; Sasaki et al., 2007). This present study reports for the first time that pretreatment of the human renal cells with plaunotol could inhibit the toxic effect caused by doxorubicin. As shown here, such a protection of plaunotol occurred only in the cells pretreated with plaunotol for at least 9 h (Figures 15 and 16). This finding suggests that the plaunotol manipulate the cytotoxic mode of doxorubicin via a process requiring time. The hydrophobic structure of plaunotol causes a poor permeability into cell membrane and might participate in the time-requiring protection of plaunotol. *In vivo* study has demonstrated that after oral administration, plaunotol needs time for 2 h to reach the highest plasma concentration (Komai et al., 1985).

Bcl-2 family protein members have attracted the most attention in the regulation of anti-apoptotic and pro-apoptotic pathways (Burlacu, 2003; Chipuk et al., 2008; Chipuk et al., 2010; Ola, Nawaz, and Ahsan, 2011; Youle and Strasser, 2008). Among them, a short half-life survival protein, Mcl-1 is the one most frequently reported to play a vital role in apoptotic cell death induced by doxorubicin (Katoh et al., 1998; Lestini et al., 2009; Lopez-Royuela et al., 2010; Wang et al., 2004; Wirth et al., 2005). In order to clarify the mechanism by which plaunotol prepared cells to survive in a doxorubicin-mediated toxic condition, Mcl-1 expressions in response to

plaunotol treatment were determined. Our results indicated that plaunotol caused Mcl-1 up-regulation in dose- and time-dependent manners (Figure 17). The above-mentioned role of Mcl-1 in doxorubicin-induced cell death was confirmed by the observation that treatment with doxorubicin of these renal cells resulted in a reduction of Mcl-1 level (Figure 13) which was correlated with the reduction in cell viability (Figure 11). Indeed, up-regulation of Mcl-1 protein in renal cells has been shown to be the main mechanism in protection of the cells against various death stimuli such as cisplatin, indomethacin and radiocontrast agents (Gong et al., 2010; Liu et al., 2010; Ou et al., 2009; Yang et al., 2007). This study also demonstrates that overexpression of Mcl-1 in human renal cells sufficiently inhibited doxorubicin-induced apoptosis. Therefore, it is possible that the increased level of Mcl-1 after plaunotol pretreatment can overcome the Mcl-1 reduction caused by doxorubicin and subsequently prevent cell death.

Plaunotol gradually increases Mcl-1 in human renal cells in time-dependent manner. The highest level of Mcl-1 had been observed in the cell treated with plaunotol for 9 h (Figures 17A and B). Similar up-regulated pattern of Mcl-1 has been depicted in treatment of proteasome inhibitor. The inhibition on protein degradation causes the sustained augmentation of Mcl-1 for 15 h (Yoon et al., 2002). It is worth noting that stimulation of protein synthesis appears with the rapidly increases and decrease level of Mcl-1 within 1 and 9 h, respective (Kuo et al., 2001). The difference of up-regulated pattern comes from the short half-life of both mRNA and protein of Mcl-1 (Kuo et al., 2001; Michels et al., 2005). In spite of a number of effective transcriptional factors including STAT3, STAT5, CREB and PU.1, none of them has been activated by plaunotol (Akgul, 2009; Fu et al., 2005). Thus that plaunotol induce

the increment of Mcl-1 in human renal cells possibly through the inhibition on protein degradation.

Mcl-1 is a short half-life anti-apoptotic protein which is degraded via ubiquitin-proteasome pathway. The presence of reactive oxygen species (ROS) activates the ubiquitination of Mcl-1 sequential with the recognition and degradation by proteasome (Blatt et al., 2009; Michels et al., 2005). Here in, DMNQ was selected to generate superoxide anion ($O_2^{\cdot -}$) in order to mimic the production of cellular ROS at normal condition. Preparing the cells with antioxidant and plaunotol significantly prevented the reduction of Mcl-1 induced by ROS in human renal cells (Figure 19). In order to demonstrate the relationship between Mcl-1 and ROS level in the cells treated with plaunotol, the capability of plaunotol to decrease basal cellular ROS in human renal cells was notified after the incubation of plaunotol for 3 h (Figure 20). This antioxidant activity of plaunotol was presented early before the up-regulation of Mcl-1 in human renal cells that were treated with plaunotol for 6 h (Figure 17). These findings support that plaunotol gradually preserves Mcl-1 in human renal cells through its antioxidant activity.

The up-regulation of Mcl-1 level in various cells has been demonstrated to relate with the expression of cyclooxygenase2 (COX2) (Chen et al., 2010; Choi et al., 2005; Kuo, Chi, and Liu, 2005; Lin et al., 2010). COX2 and PEG2 mediate PI3K/Akt which sequentially increases the level of Mcl-1 through both stimulation of transcription and inhibition of degradation (Chen et al., 2010; Lin et al., 2010; Maurer et al., 2006). Depended on the stimulation of COX2 synthesis in gastric cells, plaunotol might increase level of COX2 sequential with the overexpression of Mcl-1

in human renal cells (Fu et al., 2005). Additionally, COX2 plays a crucial role to maintain normal function of renal cells (Brater et al., 2001; Hao et al., 1999).

This work is the first to clarify the antioxidant mechanism of plaunotol. ROS scavenging activity of plaunotol was evaluated through the interaction between free radicals and plaunotol in non-cell base assay. Although many natural extracts present a high capability to directly react with free radical molecules, ROS scavenging activity of plaunotol was barely detected in DPPH and ORAC assay. Most of natural antioxidants compose with a phenolic structure which potentially donate hydrogen molecule to free radical (Kolak et al., 2009; Rice-Evans, Miller, and Paganga, 1996). The absence of this aromatic ring on the structure of plaunotol might lead to low property of ROS scavenger. However, the competency to demolish three hazard ROS including superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot}$) was demonstrated in human renal cells treated with plaunotol for 3-6 h (Figures 21-23). The reduction of superoxide radical in human blood cells also has been reported in patients who administer plaunotol for 7 days (Okabe et al., 1995). Together, antioxidant activity of plaunotol should come from the induction of cellular defensive mechanism not the directly chemical reaction.

Feeding animal with plaunotol increases thiol (SH) molecule in gastric mucosal cells (Ohta et al., 2005). Correspondent with the results in this work, glutathione (GSH) content in human renal cells was increased after treatment of plaunotol in time- and dose-dependent manner. Nevertheless, the alteration of antioxidant enzymes including superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx) were not found after the treatment of human renal cells with plaunotol. The underlying mechanisms of plaunotol-mediated GSH level in human

renal cells should be further investigated. Beside the activation on the transcription of γ-glutamylcystein synthase (γ-GCS) through Nf-κB transcriptional factor, plaunotol might stimulate the turnover of GSH from oxidized GSH, GSSG (Fu et al., 2005; Lu, 2009). The increase of thiol molecule (SH) stimulates the recovery of GSH from the reaction between thiol-protein and GSSH as following equation (Lu, 2009; Ohta et al., 2005).

Cellular thiol-disulfide equilibrium: P-SSG + GSH $\rightleftharpoons$ P-SH + GSSH

In patient with renal injury, GSH is a cellular antioxidant molecule that has been shown to relate with the normal kidney function (Lash, 2005; Santangelo et al., 2004). Although the largest amount of biological GSH is produced in liver, the ability to produce and import GSH inside renal cells is also demonstrated (Lash, 2005; Moldeus, Ormstad, and Reed, 1981). The increase of GSH content in renal cells effectively prevents cell death induced by various toxic agents (Abul-Ezz, Walker, and Shah, 1991; Lash, Putt, and Matherly, 2002; Okuda et al., 2000). Moreover, the low level of GSH results in down-regulation of anti-apoptotic protein (Celli et al., 1998; D'Alessio et al., 2004; Friesen, Kiess, and Debatin, 2004). Celli and co-workers presented that GSH was responsible for the degradation of Bcl-2, an anti-apoptotic protein (Celli et al., 1998). In this study, the up-regulation of Mcl-1, a Bcl-2 family protein in human renal cells was observed after the increase of cellular GSH induced by plaunotol (Figure 27). These results imply that plaunotol up-regulates Mcl-1 level as a sequential result from protein preservation by augmented GSH level in human renal cells.

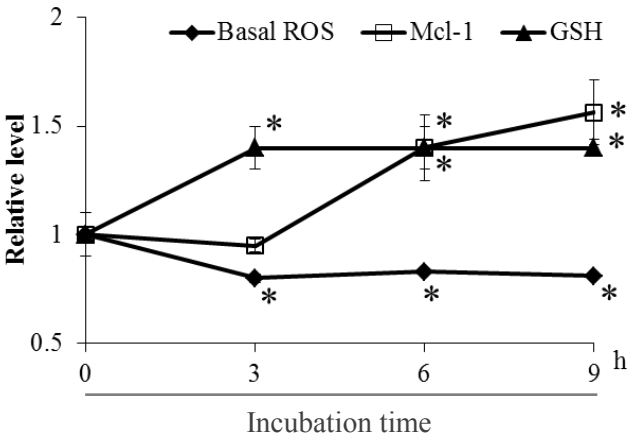


Figure 27. The time-dependent effect of plaunotol on the alteration of GSH, basal ROS and Mcl-1 in human renal cells. The graph shows the sequential alteration of glutathione (GSH), basal reactive oxygen species (ROS) and Mcl-1 level after incubation of HK-2 with 40 μM palunotol for 0-9 h. Values are means on triplicate samples±S.D. *, $p < 0.05$ versus non-treated control.

Both non-small cell lung carcinoma, H460 and human melanoma cells, G361 have been reported to show chemotherapeutic resistance through Mcl-1 mediation (Cheng et al., 2010; Chetoui et al., 2008; Khodadoust et al., 2009; Wang et al., 2008). Interestingly, the selective protection by plaunotol in renal cells, but not in H460 and G361 cells was indicated in this study (Figures 25 and 26). Plaunotol did not inhibit doxorubicin-induced apoptosis in cancer cells and could enhance the apoptotic response in melanoma cells. Although the definitive explanation for the selectivity of this compound in normal and tumor cells is still elusive, such selectivity may be caused by the direct anti-cancer property of plaunotol as demonstrated in gastric and colon cancer cells. The increase of pro-apoptotic protein, Bax without alteration of

other Bcl-2 family proteins has been demonstrated in cancer cell treated with plaunotol (Yamada et al., 2007; Yoshikawa et al., 2009).

In conclusion, plaunotol can prevent doxorubicin-induced apoptosis in human renal cells via increasing Mcl-1 protein (Figure 26). The gradually up-regulated Mcl-1 corresponds to antioxidant activity and higher cellular GSH content induced by plaunotol. This study shows the possibility of initiating the development of plaunotol as chemoprotective therapy for doxorubicin-based treatment. Nevertheless, the protective effect of plaunotol in prevention on other nephrotoxic drugs such as cisplatin and indomethacin that mediate the down-regulation of Mcl-1 level should be more evaluated. The selectivity of plaunotol on the protection only normal cells without alteration on chemotherapeutic efficacy also need more clarification in both in vitro and in vivo study.

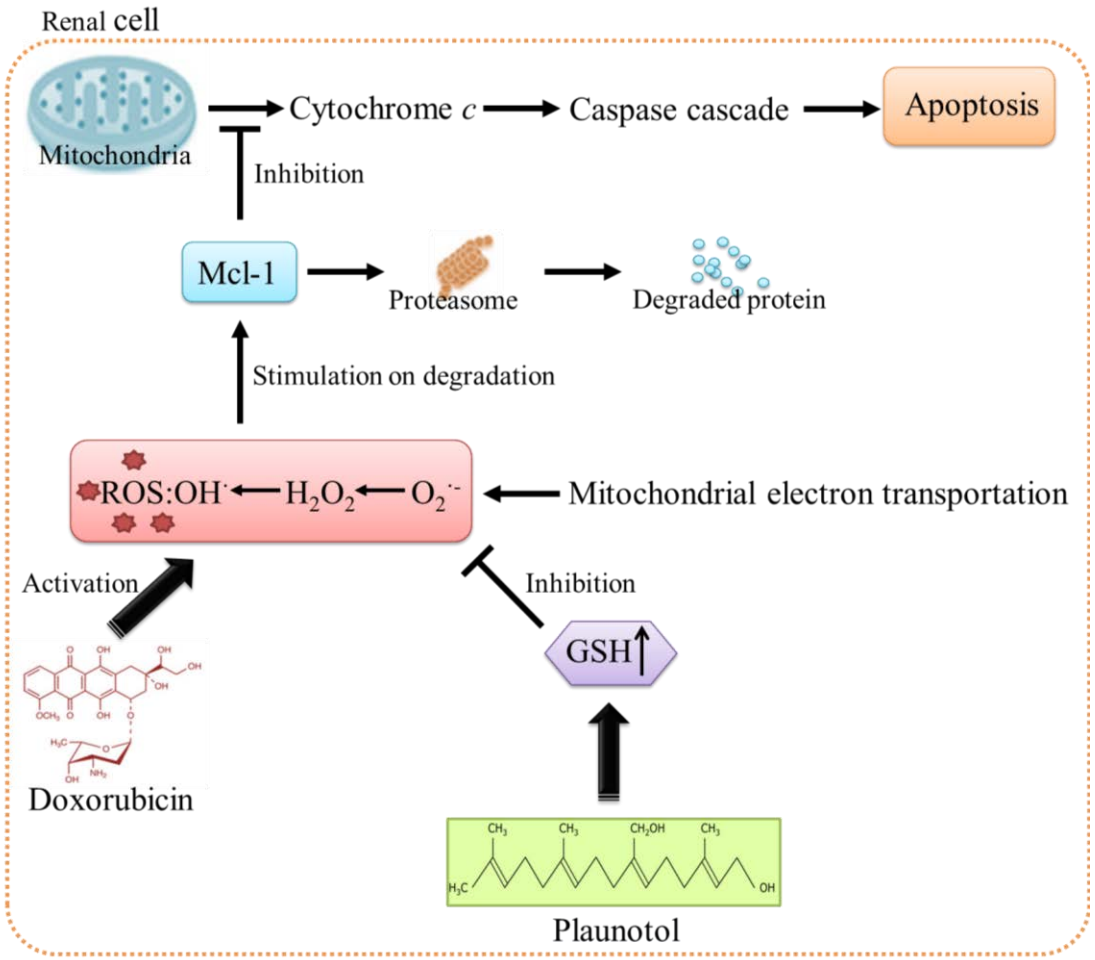


Figure 28. Proposed mechanism of the protective activity of plaunotol in human renal cells against doxorubicin toxicity. The reduction of Mcl-1 in human renal cells treated with doxorubicin results in apoptotic cell death. Plaunotol prevents doxorubicin-induced apoptosis through its antioxidant activity. Plaunotol increases the level of glutathione (GSH) which in turn inhibits Mcl-1 degradation induced by ROS.

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APPENDICES

Appendix A

SUPPLEMENTARY MATERIAL

1. Cytotoxicity effect of plaunotol on human renal cells

The non-toxic concentrations that had been used for the investigation of the protective effect of plaunotol in human renal cells were examined. After the incubation of HK-2 cells with 0-100 μM plaunotol for 24 h, MTT assay revealed that there was no significant reduction of %cell viability in the cells treated with plaunotol at concentration of l0-40 μM (Figure 27).

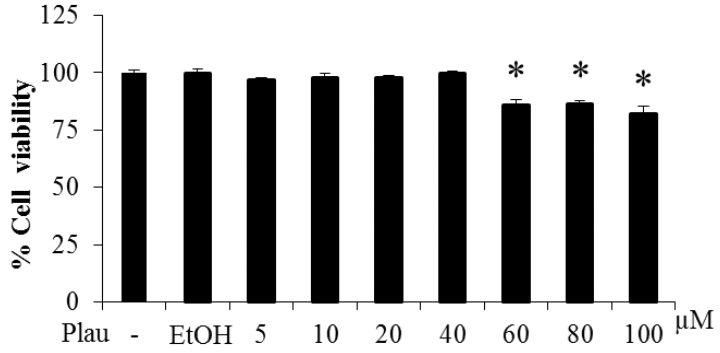

Figure 29. Cytotoxicity of plaunotol in human renal cells. MTT assay was used to investigate the cytotoxicity of plaunotol in HK-2 cells. After treatment HK-2 with plaunotol (Plau) and 0.25% ethanol (EtOH: as a solvent of plaunotol) for 24 h, the reduction of %cell viability were significantly shown in the cells incubated with 60-100 μM plaunotol. Values are means on triplicate samples±S.D. *, $p < 0.05$ versus non-treated control.

APPENDIX B

TABLE OF EXPERIMENT RESULTS

Table 2. Percentage of cell viability and apoptosis induced by 4 μM doxorubicin for 4-24 h in HK-2 cells.

Doxorubicin (h)	% Cell viability	% Apoptosis
Non-treatment	100.00±1.11	5.93±0.31
4	97.79±2.06	5.72±2.15
8	91.03±2.33*	10.86±3.74*
12	82.09±2.34*	13.75±1.69*
16	66.08±1.08*	27.09±3.34*
24	65.54±0.85*	35.70±1.39*

Each value represents mean ± S.D. of three independent experiments. *, $p <$ 0.05 versus non-treated control.

Table 3. Percentage of cell viability and apoptosis induced by 1-8 μM doxorubicin for 24 h in HK-2 cells.

Doxorubicin (μM)	% Cell viability	% Apoptosis
Non-treatment	100.00±1.52	54.92±0.16
1	78.89±0.66*	15.83±5.01*
2	77.90±3.07*	28.37±2.94*
4	66.03±2.46*	35.50±2.12*
6	62.94±1.72*	36.68±5.94*
8	62.36±0.35*	37.03±8.60*

Each value represents mean ± S.D. of three independent experiments. *, $p < 0.05$ versus non-treated control.

Table 4. Percentage of cell viability and apoptosis induced by the 4 μM doxorubicin for 24 h in non-transfected and Mcl-1-pcDNA3.1 transfected HK-2 cells.

Treatment	% Cell viability	% Apoptosis
None	100.00±0.86	5.86±0.89
Doxorubicin	65.90±0.44	32.67±1.67
+ pcDNA3.1	62.24±2.27	35.71±2.05
+Mcl-1- pcDNA3.1	76.46±3.37*#	19.94±1.03*#

Each value represents mean ± S.D. of three independent experiments. *, $p < 0.05$ versus non-transfected HK-2 cells, and #, $p < 0.05$ versus control plasmid-transfected HK-2 cells with treatment of 4 μM doxorubicin.

Table 5. Percentage of cell viability induced by 5-100 μM plaunotol for 24 h in HK-2 cells.

Plaunotol (μM)	% Cell viability
Non-treatment	100.00±1.15
5	99.70±1.89
10	96.80±1.02
20	98.03±1.62
40	97.71±1.05
60	99.49±1.12*
80	85.84±2.47*
100	86.32±1.66*

Each value represents mean ± S.D. of three independent experiments. *, $p < 0.05$ versus non-treated control.

Table 6. Percentage of cell viability and apoptosis induced by 4 μM doxorubicin in HK-2 cells with pretreatment of 40 μM plaunotol for 1-12 h.

Treatment	% Cell viability	% Apoptosis
None	100.00±3.13	4.92±0.16
Doxorubicin	67.05±1.43*	34.04±3.45*
+Plaunotol 1 h	68.36±2.33*	34.49±6.40*
+Plaunotol 3 h	66.75±1.93*	31.68±7.89*
+Plaunotol 6 h	70.69±1.12*	30.48±5.13*
+Plaunotol 9 h	78.44±4.42*#	19.30±2.68*#
+Plaunotol 12 h	80.83±0.05*#	18.96±1.06*#

Each value represents mean ± S.D. of three independent experiments. *, $p < 0.05$ versus non-treated control. #, $p < 0.05$ versus doxorubicin-treated control.

Table 7. Percentage of cell viability and apoptosis induced by 4 μM doxorubicin in HK-2 cells with pretreatment of 5-40 μM plaunotol for 9 h.

Treatment	% Cell viability	% Apoptosis
None	100.00±0.35	5.03±0.26
Doxorubicin	69.30±0.47*	31.49±3.41*
+Plaunotol 5 μM	65.71±3.08*	30.84±4.25*
+Plaunotol 10 μM	67.38±2.15*	30.40±1.40*
+Plaunotol 20 μM	76.38±0.50*#	25.53±2.22*
+Plaunotol 40 μM	84.99±2.55*#	18.65±1.80*#

Each value represents mean ± S.D. of three independent experiments. *, $p < 0.05$ versus non-treated control. #, $p < 0.05$ versus doxorubicin-treated control.

Table 8. Relative fluorescence of DHE induced by 10 μM DMNQ in HK-2 cells with pretreatment of MnTBAP or plaunotol.

Treatment	Relative fluorescence (DHE)
None	1.00±0.01
DMNQ	1.67±0.03*
+MnTBAP 50 μM	1.17±0.02*#
+Plaunotol 40 μM	1.28±0.11*#

Each value represents mean ± S.D. of three independent experiments. *, $p < 0.05$ versus non-treated control. #, $p < 0.05$ versus DMNQ-treated control.

Table 9. Relative fluorescence of H_2DCF-DA in HK-2 cells with treatment of antioxidant or 40 μM plaunotol for 1-9 h.

Treatment	Relative fluorescence (DCF)
None	1.00±0.10
GSH	0.78±0.02*
NAC	0.70±0.03*
Plaunotol 1 h	0.91±0.02
Plaunotol 3 h	0.80±0.02*
Plaunotol 6 h	0.83±0.01*
Plaunotol 9 h	0.81±0.01*

Each value represents mean ± S.D. of three independent experiments. *, $p < 0.05$ versus non-treated control.

Table 10. Relative fluorescence of H_2DCF-DA in HK-2 cells with treatment of antioxidant or 5-10 μM plaunotol for 6 h.

Treatment	Relative fluorescence (DCF)
None	1.00±0.13
GSH	0.75±0.01*
NAC	0.71±0.09*
Plaunotol 5 μM	0.91±0.03
Plaunotol 10 μM	0.86±0.04
Plaunotol 20 μM	0.84±0.04*
Plaunotol 40 μM	0.80±0.01*

Each value represents mean ± S.D. of three independent experiments. *, $p < 0.05$ versus non-treated control.

Table 11. Relative fluorescence of DHE induced by 10 μM DMNQ in HK-2 cells with pretreatment of antioxidant or 40 μM plaunotol for 1-6 h.

Treatment	Relative fluorescence (DHE)
None	1.00±0.02
DMNQ	1.25±0.03*
+GSH	0.99±0.03#
+NAC	1.02±0.03#
+Plaunotol 1 h	1.17±0.14*
+Plaunotol 3 h	1.04±0.10#
+Plaunotol 6 h	0.99±0.09#

Each value represents mean ± S.D. of three independent experiments. *, $p < 0.05$ versus non-treated control. #, $p < 0.05$ versus DMNQ-treated control.

Table 12. Relative fluorescence of DHE induced by 10 μM DMNQ in HK-2 cells with pretreatment of antioxidant or 5- 40 μM plaunotol for 6 h.

Treatment	Relative fluorescence (DHE)
None	1.00±0.02
DMNQ	1.27±0.03*
+GSH	1.04±0.01#
+NAC	1.06±0.02#
+Plaunotol 5 μM	1.26±0.10*
+Plaunotol 10 μM	1.24±0.06*
+Plaunotol 20 μM	1.16±0.06*
+Plaunotol 40 μM	0.95±0.05#

Each value represents mean ± S.D. of three independent experiments. *, $p < 0.05$ versus non-treated control. #, $p < 0.05$ versus DMNQ-treated control.

Table 13. Relative fluorescence of H_2DCF-DA induced by 100 μM H_2O_2 in HK-2 cells with pretreatment of antioxidant or 40 μM plaunotol for 1-6 h.

Treatment	Relative fluorescence (DCF)
None	1.00±0.13
H_2O_2	2.74±0.41*
+GSH	1.63±0.10*#
+NAC	1.48±0.11*#
+Plaunotol 1 h	2.41±0.32*
+Plaunotol 3 h	2.25±0.03*#
+Plaunotol 6 h	2.15±0.23*#

Each value represents mean ± S.D. of three independent experiments. *, $p < 0.05$ versus non-treated control. #$p < 0.05$ versus H_2O_2-treated control.

Table 14. Relative fluorescence of H_2DCF-DA induced by 100 μM H_2O_2 in HK-2 cells with pretreatment of antioxidant or 5-40 μM plaunotol for 6 h.

Treatment	Relative fluorescence (DCF)
None	1.00±0.01
H_2O_2	2.94±0.54*
+GSH	1.71±0.13*#
+NAC	1.56±0.13*#
+Plaunotol 5 μM	2.34±0.09*
+Plaunotol 10 μM	2.34±0.09*
+Plaunotol 20 μM	2.32±0.09*
+Plaunotol 40 μM	2.13±0.08*#

Each value represents mean ± S.D. of three independent experiments. *, $p < 0.05$ versus non-treated control. #$p < 0.05$ versus H_2O_2-treated control.

Table 15. Relative fluorescence of HPF induced by Fe^{2+}+ 100 μM H_2O_2 in HK-2 cells with pretreatment of antioxidant or 40 μM plaunotol for 1-6 h.

Treatment	Relative fluorescence (HPF)
None	1.00±0.02
Fe^{2+}+H_2O_2	1.76±0.01*
+GSH	0.97±0.05#
+NAC	0.99±0.07#
+Plaunotol 1 h	1.72±0.12*
+Plaunotol 3 h	1.62±0.09*
+Plaunotol 6 h	1.59±0.05*#

Each value represents mean ± S.D. of three independent experiments. *, $p < 0.05$ versus non-treated control. #$p < 0.05$ versus Fe^{2+}+H_2O_2-treated control.

Table 16. Relative fluorescence of HPF induced by Fe^{2+}+ 100 μM H_2O_2 in HK-2 cells with pretreatment of antioxidant or 5-40 μM plaunotol for 6 h.

Treatment	Relative fluorescence (HPF)
None	1.00±0.02
Fe^{2+}+H_2O_2	1.77±0.01*
+GSH	0.99±0.08#
+NAC	0.93±0.04#
+Plaunotol 5 μM	1.75±0.05*
+Plaunotol 10 μM	1.83±0.02*
+Plaunotol 20 μM	1.63±0.05*
+Plaunotol 40 μM	1.51±0.09*#

Each value represents mean ± S.D. of three independent experiments. *, $p < 0.05$ versus non-treated control. #$p < 0.05$ versus Fe^{2+}+H_2O_2-treated control.

Table 17. Relative GSH induced by N-acetylcystein (NAC) or 40 μM plaunotol for 1-6 h in HK-2 cells.

Treatment	Relative GSH content
None	1.00±0.02
NAC	1.49±0.19*
Plaunotol 1 h	1.28±0.10
Plaunotol 3 h	1.40±0.10*
Plaunotol 6 h	1.40±0.04*

Each value represents mean ± S.D. of three independent experiments. *, $p < 0.05$ versus non-treated control.

Table 18. Relative GSH induced by N-acetylcystein (NAC) or 5-40 μM plaunotol for 6 h in HK-2 cells.

Treatment	Relative GSH content
None	1.00±0.06
NAC	1.32±0.11*
Plaunotol 5 μM	1.12±0.01
Plaunotol 10 μM	1.18±0.10
Plaunotol 20 μM	1.25±0.17*
Plaunotol 40 μM	1.37±0.11*

Each value represents mean ± S.D. of three independent experiments. *, $p < 0.05$ versus non-treated control.

Table 19. Percentage of cell viability and apoptosis induced by 4 μM doxorubicin in H460 cells with pretreatment of 40 μM plaunotol.

Treatment	% Cell viability	% Apoptosis
None	100.00±1.62	1.57±0.47
Plaunotol	89.49±3.36*	2.25±1.25*
Doxorubicin	76.75±1.39*	20.43±1.30*
+Plaunotol 9 h	75.55±0.66*	19.59±1.88*

Each value represents mean ± S.D. of three independent experiments. *, $p < 0.05$ versus non-treated control. #, $p < 0.05$ versus doxorubicin-treated control.

Table 20. Percentage of cell viability and apoptosis induced by 4 μM doxorubicin in G361 cells with pretreatment of 40 μM plaunotol.

Treatment	% Cell viability	% Apoptosis
None	100.00±0.70	4.77±0.96
Plaunotol	82.87±1.34*	11.13±0.99*
Doxorubicin	71.18±3.01*	22.39±3.01*
+Plaunotol 9 h	59.87±2.75*#	27.03±3.33*#

Each value represents mean ± S.D. of three independent experiments. *, $p < 0.05$ versus non-treated control. #, $p < 0.05$ versus doxorubicin-treated control.

VITA

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