國立臺灣大學生命科學院生化科技系

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Thioredoxin 與化療藥物治療後產生癌細胞抗藥性之關聯性

The Possible Role of Thioredoxin in causing Drug
Resistance following Chemotherapy

胡景涵

Ching-Han Hu

指導教授：莊榮輝 博士

闕壯卿 博士

Advisor: Rong-Huay Juang, Ph.D.

Chuang-Chin Chiueh, Ph.D.

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　　本論文係 胡景涵 君（學號 R00B22036 ）在國立臺灣大學生化科技學系完成之碩（博）士學位論文，於民國 102 年 6 月 24 日承下列考試委員審查通過及口試及格，特此證明

口試委員：　關杰雄　莊榮輝 （簽名）

（指導教授）

黃楓婷.

許壽齡　　楊健志

系主任、所長　　黃慶璨 （簽名）

誌謝

　　從一開始進入這個所到現在，兩年的時光就這樣匆匆的過去了。由於大學時是念護理系，因此為了要轉進入研究的領域，花費了一年的時間終於讓我考上了，抱著好奇及期待的心來到莊榮輝老師的實驗室。碩一的時候由於什麼都還不懂，任何實驗也都沒接觸過，在學長界帶領下學了很多實驗技巧，也在修課中學了很多知識，但是當時對於自己的實驗方向卻是一片茫然，直到碩一下有去北醫和關壯卿老師討論後，才漸漸有了眉目。

　　在碩一下及碩二上的時間，對我來說是一段很辛苦的過程，在試著用不同方法做實驗時一開始總是失敗再失敗，有時真的感覺快被實驗失敗的挫折打倒。感謝莊榮輝老師與關壯卿老師，在每次的討論中都了我寶貴的建議及提供我思考實驗方向的建議；感謝承佑學長及惠玉姊在我什麼都不懂時，帶我學會了最基礎的實驗，並與我討論；感謝玉米學姐及蛋姊總是在我實驗失敗的時候，幫我查看實驗條件，尋找可能讓實驗失敗的原因，每次都讓我在失敗中感覺燃起了一線希望；感謝明孝學長教會我很多實驗技術及小撇步，常常要幫在我實驗出現問題時出手救急，也常常一起實驗做到很晚，因此在實驗室天南地北的聊天；感謝于嫩總是在一些小地方幫我的忙，也會提醒健忘的我很多事情；另外還要感謝張麗冠老師，提供螢光顯微鏡，使我的實驗能夠進行。

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Abstract

The possible Role of Thioredoxin

in Causing Drug Resistance following Chemotherapy

Ching-Han Hu

Department of Biochemical Science and Technology College of Life Science, National Taiwan University

Backgrounds

Neuroblastoma is a kind of children cancer that has a relatively high mortality, recurrence and metastasis; drug resistance is frequently seen after the treatment with chemotherapy (Marvis, 2010). According to the theory of cancer stem cell (CSC), the reason why cancer usually recurs and metastasizes is due to the CSC exists in the tumor which can even survive and grow into a new tumor after chemotherapy (Jordan, Guzman, and Noble, 2007).

After a new discovery of our team (Andoh, Chock, and Chiueh, 2002), a CD-133 positive and Trx-expressed CSC-like cells (N cells) were isolated from the pre-conditioned human SH-SY5Y cells (unpublished observations of Chiueh et al). N cells produced a solid tumor but not by its wild type control cells (D cells) in nude mice. D cells are associated with low Trx expression and also sensitive to oxidative injury and related apoptotic cell death.

Objective

To understand whether Trx expression affects the relationship between the preconditioned N cells and resistance to chemotherapy; to investigate the redox cycling role of Trx in mediating chemo-resistance

Methods

Trx expression in control D cells and pre-conditioned N cells were measured by the Western blotting.　Etoposide (0 to 83 μM)-induced apoptotic changes and cell viability in D and N cells were assayed by Hoechst 33258 nuclear staining and Trypan blue exclusion assay, respectively.　　The redox cycling of Trx was blocked by a Trx reductase inhibitor 1-Chloro-2,4-dinitrobenzene (DNCB (50 μM) in N cells.　Cell morphology in control D cells after etoposide treatment (0 to 83 μM) for 24 hours was imaged by using fluorescence microscopy.

Results

First, this experiment was to compare the Trx expression of the pre-conditioned N cells with the control D cells.　The result indicates that the Trx expression of N cells was 3 times greater than D cells.　Etoposide-induced apoptosis in the pre-conditioned N cells were less than that in the control D cells by 27-fold.　Next, after the co-treatment of N cells with 50 μM DNCB to block the redox cycling of Trx, the apoptosis cell percentage of DNCB-treated N cells was close to the control D cells. The present results show that the elevation of the Trx expression improved not only the survival rate of N cells, but also induced chemo-resistance reflected by a reduction in the apoptotic cell death caused by etoposide.　In addition, following the etoposide (83

μM) treatment, the phenotype of the D cells was changed to N cell morphology with a concurrent increase in Trx levels.   This phenomenon infers that the normal cancer cell may be mutated and altered into CSC-like cells under the stimulation of chemopherapy.
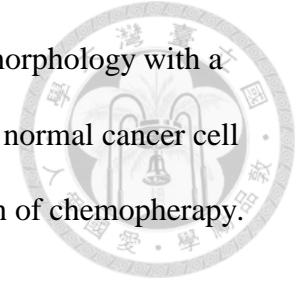
Conclusions

The expression of Trx can prevent cancer cells such as N cells from apoptotic cell death caused by etoposide.   The Trx expression and the cell morphology were altered after the treatment of etoposide in the control D cells.   The redox cycling of Trx in human neuroblastoma cancer cells may play a critical role in the development of chemo-resistance.

Key words

cancer stem cell, chemotherapy, drug resistance, etoposide, metastasis, thioredoxin

Abstract in Chinese

Thioredoxin 與化學藥物治療後產生癌細胞抗藥性之關聯性

胡景涵

國立臺灣大學生命科學院生化科技系

研究背景

　　神經纖維母細胞乃常見好發於兒童之惡性腫瘤，死亡率極高，且經治療後，仍常見復發及轉移之現象 (Marvis, 2010)。根據癌症幹細胞之理論，認為癌症之所以常見復發及轉移之現象，是由於腫瘤裡面存在著癌症幹細胞，此類細胞在經由化療藥物處理下可以繼續存活 (Jordan, Guzman, and Noble, 2007)。

　　而根據本研究團隊於 2002 年所做之實驗發現 (Andoh , Chock , and Chiueh, 2001)，經由 pre-conditioned 之 SH-SY5Y cells (N cells) 會表現 CD-133 細胞表面抗原及 Trx，相較於 wild type control cells (D cells) N cells 可形成一顆腫瘤及轉移。 D cells 之 Trx 表現量較低並且對於外界氧化壓力較敏感而較容易造成細胞凋亡之現象。

研究目的

　　希望了解 Thioredoxin 表現量是否影響癌症幹細胞與化療藥物抗藥性之間的關係，並了解 Trx 之 redox cycling 與化療藥物抗藥性之關係。

研究方法

　　希望藉由 Western blotting 來了解在 control D cell 及 pre-conditioned N cell 其 Thioredoxin 表現量的差異。並藉由 Hoechst 33258 細胞核染劑及 Trypan blue exclusion assay 來了解在加入 etoposide (0 to 83 μM) 後，兩株細胞細胞凋亡與存活率之差異。藉由螢光顯微鏡觀察觀察 control D cell 在加入 etoposide (0 to 83 μM) 作用 24 小時後，細胞型態變化情形。使用 DNCB (50 μM) 做為 Trx 之 redox cycling 的抑制劑。

結果

　　此次實驗首先比較 pre-conditioned N cells 與 control D cells 之 Trx 表現量之差異，結果顯示 N cells 之 thioredoxin 表現量確實較 D cells 高 3 倍。接著進行 etoposide 加藥實驗，經實驗計算發現，pre-conditioned N cells 要達到與 control D cells 相同之 a 細胞凋亡比率，需 27 倍的藥物劑量。再者加入 DNCB (50 μM)抑制 Trx 之 redox cycling，可發現經 DNCB 處理之 N cells 其細胞凋亡之比率與 control D cells 相近。此結果顯示 thioredoxin 表現量差異的確會影響 N cells 之存活率，並且影響 etoposide 所產生之細胞凋亡導致細胞對化療藥物產生抗藥性之現象。另外在進行加藥實驗時發現，D cells 在高劑量化療藥物 (83 μM etoposide) 作用時，經由顯微鏡觀察，發現其細胞型態改變且與 N cell 之型態相似，並且 Trx 表現量上升。因此推測可能在化療藥物作用下，normal cancer cell 會經由刺激而突變或是篩選成與癌症幹細胞相似之細胞。

結論

Trx 表現量確實會避免癌症細胞對化療藥物 etoposide 所導致之細胞凋亡產生抗藥性。並且 control D cells 在 etoposide 作用之下，Trx 表現量及細胞型態皆有改變。Trx 之 redox cycling 在化療藥物產生抗藥性中扮演重要的角色。

關鍵字

癌症幹細胞、化療、抗藥性、 etoposide、轉移、 thioredoxin

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Abbreviations

CSC	cancer stem cell
Trx	thioredoxin
TrxR	thioredoxin reductase
DNCB	1-Chloro-2,4-dinitrobenzene
SH-SY5Y	neuroblastoma

Text

X

Chapter 1: Preface

Rationale

The mortality of neuroblastoma is extremely high; this cancer attacks in younger children, recurrence and metastasis are often happened (Marvis, 2010). To search for an effective method for curing these kinds of cancer is the research goal of many biomedical researchers. The actual mechanism of tumorigenesis is not currently known whether drug-resistant cancer stem cells play a key role in metastasis and / or recurrence of cancers reminds to be studied (Huntly and Gilliand, 2005). According to the theory of cancer stem cell (CSC), CSCs are usually survive and growing into a new tumor after chemotherapy and metastasis to other organs (Jordan, Guzman, and Noble, 2007). Based on a previous report before (Andoh, Chock, and Chiueh, 2001), thioredoxin (Trx) expression is higher than normal cells in some preconditioned SH-SY5Y cancer cells which are less sensitive by 30-fold than the wild type neuroblastoma cells (D cells) to cytotoxic insults evoked by oxidative stress and neurotoxin as well. Recently, CD-133 positive CSCs were isolated from the preconditioned SH-SY-5Y cells and purified by cell sorting procedure to reach a 90% homogeneity (unpublished observations of Chiueh et al). These CD-133 positive and Trx-enriched CSCs (N cells) produced tumor in vivo in nude mice which was

aggressive in metastasis to other internal organs (Chiueh et al., in preparation).   In

theory, these Trx-enriched cancer stem cells may be highly resistant to most of

anti-cancer agents leading to drug resistance in chemotherapy and recurrent metastasis

with a poor treatment outcome in clinic.
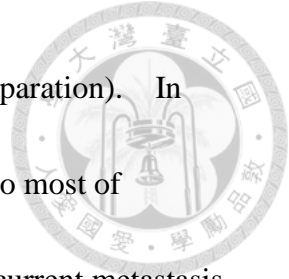
Working Hypothesis

A persistent induction of the redox protein Trx may lead to drug resistance in

the chemotherapy of cancers.

Research Goal

The current research goal is to understand the role of the inducible redox

protein thioredoxin in the chemo-resistance between pre-conditioned N cells and control

D cells following the treatment of a chemotherapeutic agent etoposide.

Chapter 2: Literature Review

2.1 Neuroblastoma

Neuroblastoma is an extracranial solid tumor that usually occurs in early childhood with high mortality (Maris et al., 2007).   It is one of the few human malignancies that demonstrate spontaneous regression from an undifferentiated state to a completely benign cellular appearance (Bénard et al., 2008).   Neuroblastoma cells are derived from the neural crest and usually arise in the adrenal medulla or along the sympathetic chain of the sympathetic nervous system; it usually originates in the adrenal gland but can also develop in nerve tissues of the neck, chest, abdomen, or pelvis (Israel et al., 1994).   It is stratified into three risk categories: low, intermediate, and high risk.   Low-risk disease is common in infant and usually has good outcomes with observation only or surgery, whereas high-risk disease is difficult to treat successfully despite intensive multi-agent induction chemotherapy, radiotherapy, surgery and 13-cis retinoic acid (Brodeur, 2003).   High-risk patients whom treated with chemotherapy usually have an inadequate response and the outcome is very poor with relapse and metastasis (Marvis, 2010). It is highly likely that metastasis of neuroblastoma cells may be caused by CSCs.

2.2 Etoposide and topoisomerase II

Etoposide is a podophyllotoxin which has been developed in 1966 (Slevin, 1991) and is currently used to treat acute myelioid, hodgkin's disease, lung cancer, breast cancer and ovarian cancer (Hande, 1998).   Now is one of chemotherapy drug that used to treat neuroblastoma.   DNA topoisomerases are nuclear enzymes with function of DNA replication, transcription, chromosomal segregation and DNA recombination (Froelich-Ammon, 1995).   It has two types present in all cells (i) topoisomerase I which makes single strand break (Wang, 1971) and (ii) topoisomerase II with double strands break and pass (Geller et al., 1976).

Etoposide is a topoisomerase II inhibitor used to treat cancers, it forms a ternary complex with DNA and the topoisomerase II enzyme, prevents re-ligation and causes DNA double strands break (Gordaliza et al., 2004).   Because of cancer cells are divide more rapidly, they rely on this enzymes more than healthy cells.   So that the complex of etoposide-DNA-topoisomerase II forms DNA breaks permanently and caused errors in DNA replication then ultimately triggers apoptosis of the cancer cell (Froelich-Ammon, 1995).   In the present study, Hoechst 33258 was used to imaging apoptotic changes in N cells and D cells with different Trx levels following the etoposide treatment.

2.3 Cancer Stem Cells (CSC)

2.3.1 Cancer Stem Cell Theory

According to the timeline from Huntly and Gilliand (2005), the CSC theory was first proposed by Rudolph Virchow in 1855.   Furth and kahn (1937) show that the injection of leukemia cells into nude mice can develop a solid tumor.   By this method, CSCs are found in many kinds of tumors, like brain, breast, colon, ovary, pancreas, prostate, melanoma et al. (Singh et al., 2003; Al-Hajj et al., 2003; O'Brien et al., 2007; Zhang et al., 2007; Li et al., 2007; Maitland and Collins, 2008; Schatton et al., 2008)

Craig, Monica, and Mark (2007) suggest that normal stem cells exist in human body.   Ordinarily, they differentiate into progenitors, and then circulated to the tissue and organs, turning into mature cells.   But due to the environmental change, after the mutation of the progenitors and mature cells, they will probably turn into cancer stems which will differentiate into a tumor (**Figure 1**).   It has been suggested that the transformation of normal stem cells into CSCs is because of a long-term and repetitive inflammation in some part of the tissues, which makes the environment nearby low in nutrition and oxygen.   And cells are forced into mutation to sustain the harsh condition. When the mutation proceeding to a certain degree, CSCs are formed

and will probably become a tumor (Heddleston et al., 2010).

Thus, some researchers has done a statistical analysis of a whole tumor, and found the CSCs do not account for most part but only 1% to 30% of all the cells in a tumor, with highly tentative to differentiation and mutation (Phillips, 2006).   CSCs have three capabilities (i) proliferation (ii) differentiation and (iii) self-renew which are similar to the normal stem cells, so this kind of cells is called CSCs (Tobias, Natasha, and Markus, 2009).
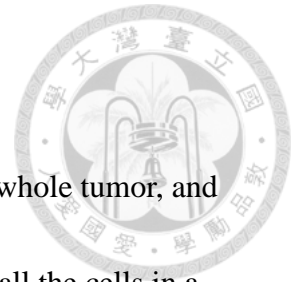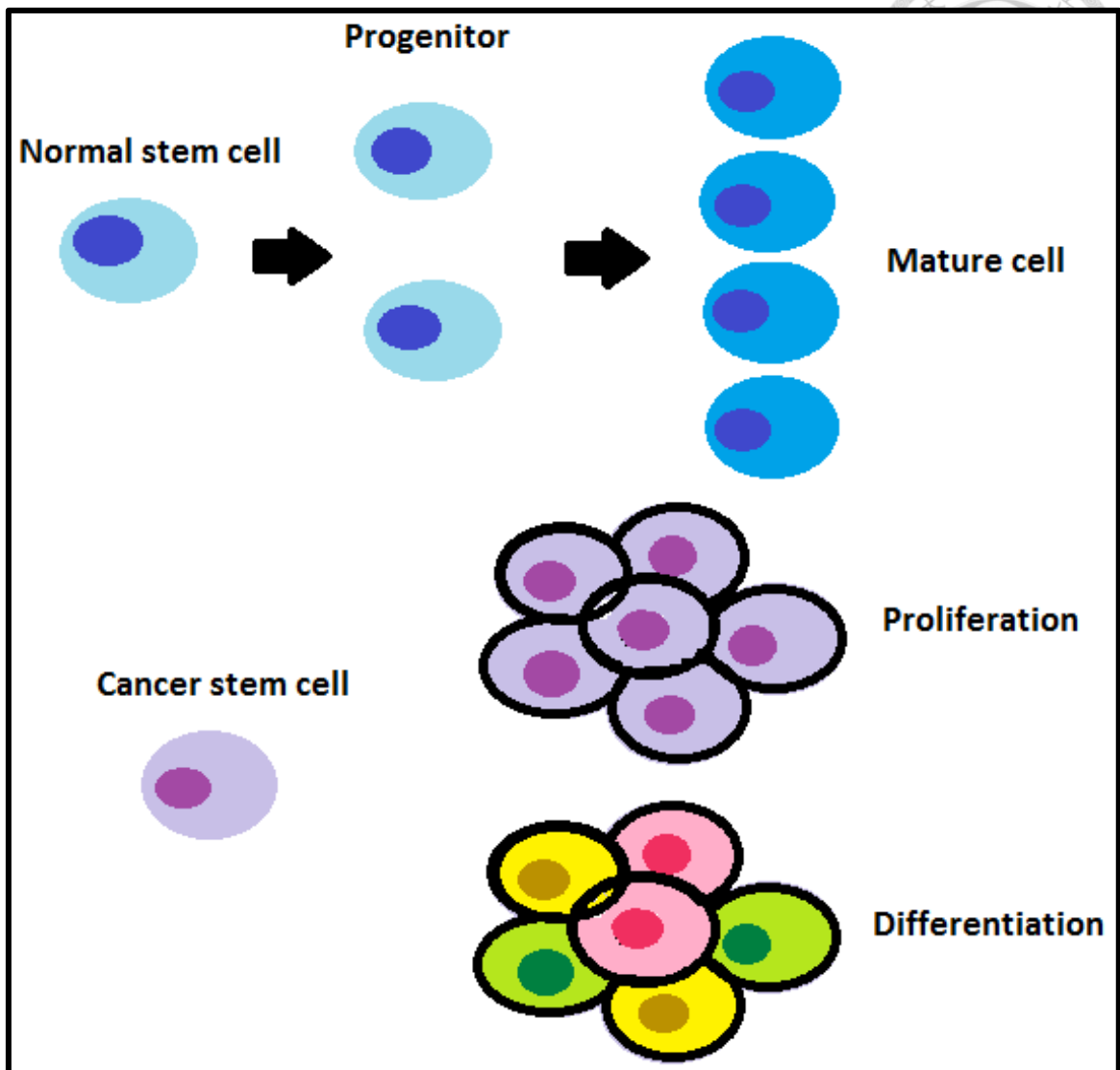
Figure 1. After mutation, normal stem cell may turn into cancer stem cell, which can probably transform to a tumor itself.

(Modified from Li, 2007 and Schatton et al., 2009)

2.3.2 Cancer stem cell markers and chemo-resistance

Surgery, chemotherapy and radiotherapy are commonly seen and arranged together in the treatment of cancers.　But recurrence and metastasis are usually happen in many cancer cases.　As a result, some researchers have proposed a theory that even though cancer cells are killed, CSCs in the tiny part of a tumor cannot be totally eliminated (**Figure 2**).　Moreover, the center of the tumor will become lack of oxygen and nutrition, which stimulates these cells into drug-resistance and hinders the treatment (Craig, Monica, and Mark, 2007).　So it is widely suggested that if we can find the marker of each kind of cancer to target the CSCs and kill it, especially when treatments and target are applied together, the rest of the normal cancer cells will not be able to survive.　In the meanwhile, the whole tumor will gradually disappear.　Then the cancer will be cured (Tobias, Natasha, and Markus, 2009; Fiona and Norman, 2011).

CSC markers have two significant characteristics (i) every different tumor type has different cell surface marker, but a cell surface marker can appear in different tumor types and (ii) no marker can be used in every tumor type (**Table 1**). Therefore, it is necessary to find out a marker that can be extensively used (Tobias, Natasha, and Markus, 2009).
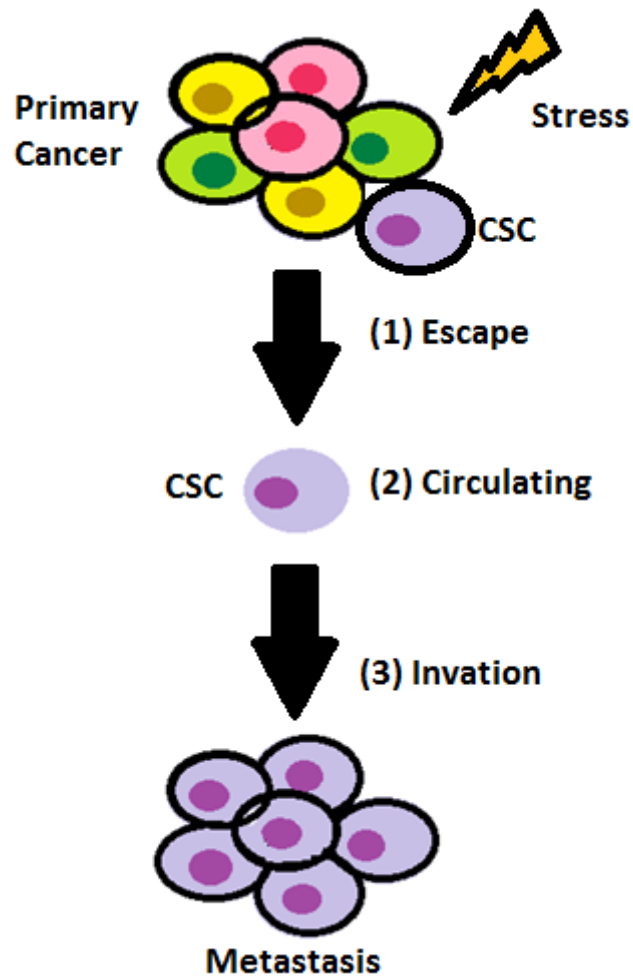
Figure 2. Cancer stem cell and chemo-resistance

(1) Escape of CSC from Primary cancer after stress induced by chemotherapy

(2) Circulating CSC in cardio-vascular system

(3) Invasion of CSC into organs. (metastasis)

Table 1. Cancer stem cell markers

Tumor type	Cell surface marker(s)	Reference
Breast	CD44+, CD24− /low Lineage−ESA+	Al-Hajj *et al*.
CNS Colon	**CD133+** CD133+	**Singh *et al*.** O'Brien *et al*.
	ESAhighCD44+ Lineage− (CD166+)	Dalerba *et al*
Ewing's	CD133+	Suva *et al*.
Head and neck	CD44+	Lineage− Prince *et al*.
Melanoma	ABCB5+	Schatton *et al*.
Ovarian	CD44+, CD117+	Zhang *et al*.
Liver	CD90+, CD45−(CD44+)	Yang *et al*.
Pancreas	CD44+, CD24+, ESA+	Li *et al*.
	CD133+	Hermann *et al*.

(Modified from Tobias, Natasha, and Markus, 2009)

2.4 Thioredoxin (Trx)

2.4.1 Trx system

Thioredoxins (Trx) are a 12 kDa redox-active proteins that contain two cysteines in the conserved center and present in all prokaryotic and eukaryotic organisms. Their redox-active sites are conserved by -Trp-Cys-Gly-Pro-Cys-Lys- (Holmgren, 1985; Eklund, Gleason, and Holmgren, 1991). The oxidized (inactive) form of Trx (Trx-S_2) can be reduced by thioredoxin reductase (TrxR) with NADPH exist, and the two cysteines formed by disulfide bond at the active site are turn into a dithiol reduced (active) form of Trx (Trx-SH_2) (Holmgren, 1995). The Trx system affect lots of biological functions such as protecting cells against apoptosis, angiogenesis, DNA replication and cell proliferation, control and regulation of numerous transcription factors activities (Andoh, Chock, and Chiueh, 2002; Laurent, Moore, and Reichard ,1964; Welsh et al., 2002; Luthman et al.,1979; Matthews et al., Abate et al., 1990). It has been observed that Trx levels are relatively high in some cancers which may link to poor treatment outcome, and was 30-times less sensitive to the neurotoxin agent, MPP^+ (Andoh, Chock, and Chiueh, 2001). And transfecting of Trx into cancer cells develops chemo-resistance (Yokomizo et al., 1995). Therefore, in the present proposal a new working hypothesis was developed and used to study the

redox role of the endogenous Trx in N cells on etoposide-induced apoptotic death and also chemo-resistance in Trx-enriched cancer cells.

2.4.2 Inhibition of Trx system

There are many kinds of Trx inhibitors or TrxR inhibitors used in clinical, such as Azelaic Acid and carmustine, respectively (Schallreuter and Wood, 1987; Schallreuter, Gleason, and Wood, 1990). Because (i) Trx is important in cancer cell survival and TrxR plays a key role in the thioredoxin system (ii) TrxR C-terminal active site can be targeted and inhibited by different electrophilic compounds. Therefore, TrxR has been measure to be a target of anticancer agents (Nguyen et al., 2006). DNCB is an electrophilic compounds and one of the TrxR inhibitor which is irreversible inhibit TrxR activity (Arner, Bjornstedt, Holmgren, 1995). This function is because of the Cys and Sec in C-terminal active site is alkylated by DNCB (Nordberg, 1998). Thus, DNCB is used to evaluate the effect of Trx in CSC-liked N cell.

Chapter 3: Materials and Methods

3.1 Materials

Human Neuroblastoma SH-SY5Y cells (D cells) were kindly provided by Dr. Carol Thiele (NCI), and the pre-conditioned Human Neuroblastoma SH-SY5Y cells (N cells) was derived from Human Neuroblastoma SH-SY5Y cells following multiple oxidative stresses. The Dulbecco's modified Eagle's medium, Etoposide, Hoechst 33258 (bisbenzimide), DNCB (1-Chloro-2,4-dinitrobenzene) and trypan blue was supplied by Sigma Aldrich (St. Louis, MO). Fetal bovine serum was purchased from BIOLOGICAL INDUSTRIES (Kibbutz Beit Haemek). The human Trx antibody and the actin antibody were obtained from abcam (Cambridge, MA) and Cell Signaling (Beverly, MA), respectively. The goat anti-rabbit IgG secondary antibody was purchased from Millipore (Billerica, MA). Protease inhibitor cocktail and RNase A were from BIONOVAS (Toronto, Ontario).

3.2 Cell Cultures

The wild type D cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum.   And the pre-conditioned Human Neuroblastoma SH-SY5Y cells (N cell) were also cultured in Dulbecco's modified Eagle's medium but 5% fetal bovine serum.   Both cell lines were incubated in 5% CO_2 without phenol red at 37℃ and changed medium every 2-3 days.

3.3 Nuclear Staining of Apoptotic Cells by Hoechest 33258

Both D and N cells were cultured to 4-5 passage and then seeded into a coated 24-well plate with different density.   The Human Neuroblastoma SH-SY5Y cells seeded with $3X10^4$ cells per well and the pre-conditioned Human Neuroblastoma SH-SY5Y cells were seeded with $4X10^4$ cells per well. After 2 to 3 days, Etoposide was added with different concentration (0.85 μM, 8.5 μM, 85 μM and 1.2% DMSO only as the control of 0 μM) and incubated for 24 hours.   At the end, both cells were washed by PBS with 3 times and then fixed with 4% paraformaldehyde in PBS at room temperature for 30 min.   After PBS washed 3 times, cells were stained by 1 μM Hoechst 33258 fluorescent dye for 5 min at room temperature.   The cell nuclear DNA was imaged and photographed with a fluorescent microscope.   The apoptotic cells were identified by the present of nuclear condensation and DNA fragmentation.
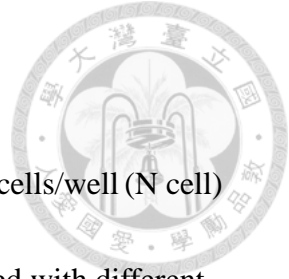
3.4 Trypan Blue Exclusion Assay

Both cells were seeded in 6-cm dish with density of $5X10^5$ cells/well (N cell) and $4X10^5$ cells/well (D cell). After 2 to 3 days, Etoposide was added with different concentration (0, 0.85, 8.5, 85μM). After incubated with Etoposide for 24 hours, cells were resuspended and then centrifuged with 900 rpm. After centrifugation, cell pellets were re-suspended with PBS and the stained cells were counted by a heamocytometer.

3.5 Western Blotting

Cells were added cell lysis bufferd 20 mM Hepes-KOH pH 7.5, 10 mM KCl, 1.5 mM MgCl$_2$, 1 mM EDTA, 1 mM dithiothreitol, and 10 μL protease inhibitor and then lysed by sonication. The protein concentration was quatified by BSA kit (Bio-Rad). Lyzed cellular protein extract (20 or 40 μg) was separated by a 20% SDS-PAGE and then transfer to polyvinylidene difluoride membrane (Millipore). After PBST (phosphate buffered saline and Tween-20) washed 3 times, polyvinylidene difluoride membrane was blocked by Gelatin- NET (0.25% Gelatin, 0.15 M NaCl, 5 mM EDTA-2Na, 0.05% Tween 20, 50 mM Tris/HCl pH8.0) for 30 min at room temperature. The target protein was probed by human Trx antibody (1:1000) and actin antibody as control (1:1000) at 4℃ overnight. After washing with PBST 3 times, the polyvinylidene difluoride membrane was incubated with 1000X dilution of secondary

goat anti-rabbit IgG antibody which was linked with horseradish peroxidase at room

temperature for 1 hour.   At the end, the target bands that bounded with horseradish

peroxidase were reacted with ECL and monitored by Chemiluminescence Imaging

System (BioSpectrum 500).   The signaling area and intensity was quantified by the

Image J.

3.6 MTT Assay for Mitochondria Activity

Both cell lines were cultured to 4-5 passage and then seeded into 24-well plate.

Both Human Neuroblastoma SH-SY5Y cells and the pre-conditioned Human

Neuroblastoma SH-SY5Y cells were seeded with 5×10^4 cells per well.   After

incubation for 1 to 2 days, etoposide was added into medium with different

concentrations (0.85, 8.5, 85 μM and DMSO only as 0 μM control) and incubated for 24

hours.   At the end, both cells were washed by PBS with 3 times, fresh medium

contained 50 μl MTT colorimetric dye (5 mg/ml) was added into each well and

incubated for 3-4 hours. After removed the supernatant, cells were washed by PBS with

3 times and resuspeded the formazen by 1mL DMSO. Each well was taken 200 μL and

measured O.D 595 by ELISA reader (Nunc, 269620).

Chapter 4: Preliminary Results

4.1 Trx expression and N cells

It is known that Trx level is higher in some cancer cell line, such as U-87MG, astroglioma; HeLa, cervical carcinoma; A549, lung carcinoma. The present study investigated whether these Trx-enriched cancer cells are resistant to most of the chemo-therapeutics. Increased Trx is also observed in the human neuroblastoma SH-SY5Y cells following pre-conditioning stress (Andoh, Chock, and Chiueh, 2001). Repeated pre-condition SH-SY5Y cells with increased levels of Trx are less sensitive to oxidative injury and cyto-toxicity. Most of the Trx-enriched and pre-conditioned cells are CD-133 positive (N cells; unpublished observation). The present study used Western blotting (**Figure 3**), and confirmed that Trx level was higher in the N cells than in the human neuroblastoma SH-SY5Y cells (D cells) by 3 times.
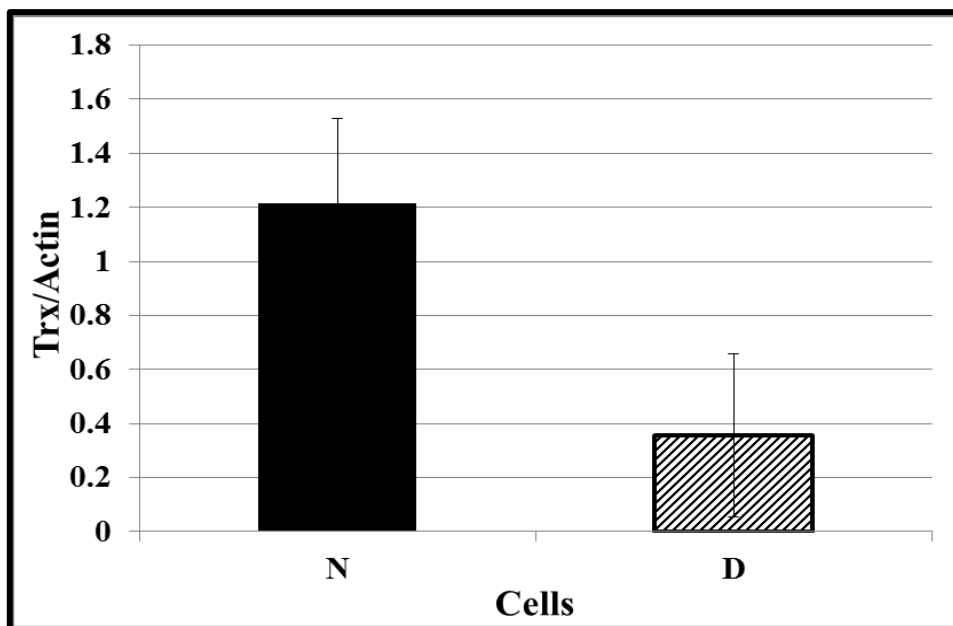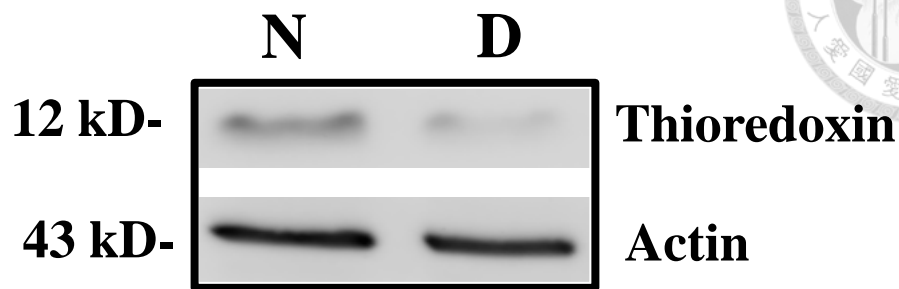
Figure 3. The expression of Trx in N cell and D cell by Western blotting

(A) Each land were loaded with 20μg of total protein extraction. The Trx expression

was monitored by anti-human Trx polyclonal antibody, and the band was in 12 kD.

Actin as loading control (43kD) was monitored by anti-human Actin monoclonal

antibody.   Human neuroblastoma SH-SY5Y cells were labeled as D cells; CD-133

positive pre-condition human neuroblastoma SH-SY5Y cells were labeled as N cells.

(B) The expression of Trx was measured by western blotting. (n = 3, mean ± SD)

4.2 Effects of etoposide

4.2.1 Apoptotic cell changes

Hoechst 33258 fluorescence dye was used to measure the apoptosis cell changes in N cells and D cells following the treatment with etoposide (0, 0.83, 8.3, 83 μM) for 24 hours. The results of fluoro-microscopy (**Figure 4**) revealed that D cells were more sensitive than N cells in etoposide-evoked apoptotic changes in nuclei (i.e, nuclear condensation, fragmentation, phenotype changed) concentration dependently. Moreover, etoposide caused a concentration-dependent apoptotic changes in the nuclear of both N cells and D cells. The result of apoptotic changes show that Trx-enriched N cells were less sensitive than D cells following etoposide treatment (**Figure 5**). The estimated in apoptotic cell changes between N cell and D cell was 1.63 ± 0.15 following the 24-hours treatment with etoposide.
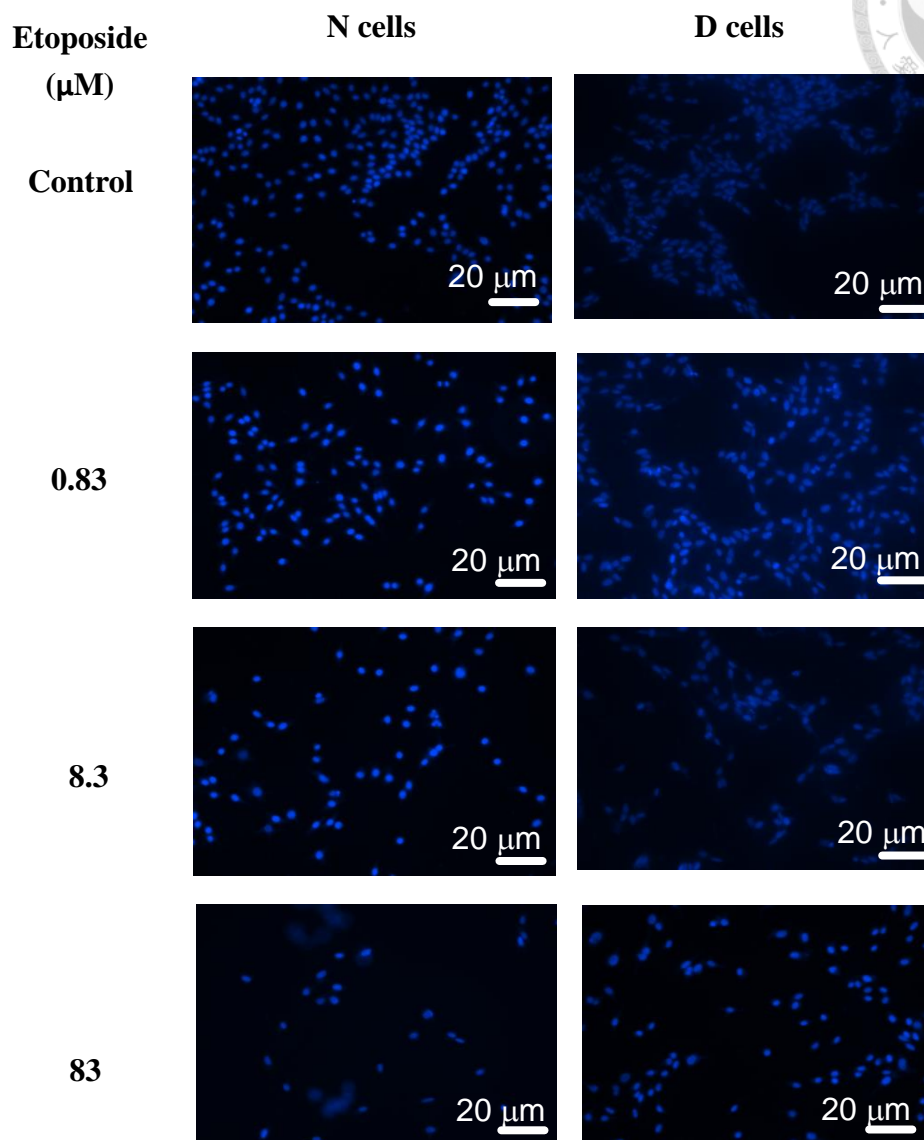
Figure 4. Effcts of Etoposide on apoptotic changes in nuclear staining by Hoechst 33258 fluorescent dye in D and N cells.

Typical results of microphotography by the fluorescence microscopy in D cells and N cells (200X) , 24 hours following the treatment with different concentration of etoposide (0.83, 8.3, 83 μM), and control media contained with 1.2% DMSO. Hoechst 33258 nuclear staining for apoptotic changes in nuclei (i.e, nuclear condensation, fragmentation, size changed). D cells: Human neuroblastoma SH-SY5Y cells

N cells: CD-133 positive pre-condition human neuroblastoma SH-SY5Y cells

Figure 5. Apoptotic cell death of D cells and N cells evoked by etoposide (0 to 83 μM). For methods, please see the legend of Figure 2.

Each well were taken 4 photos and calculated by Hoechst 33258 nuclear staining for apoptotic changes in nuclei. (n = 3, mean ± SD).
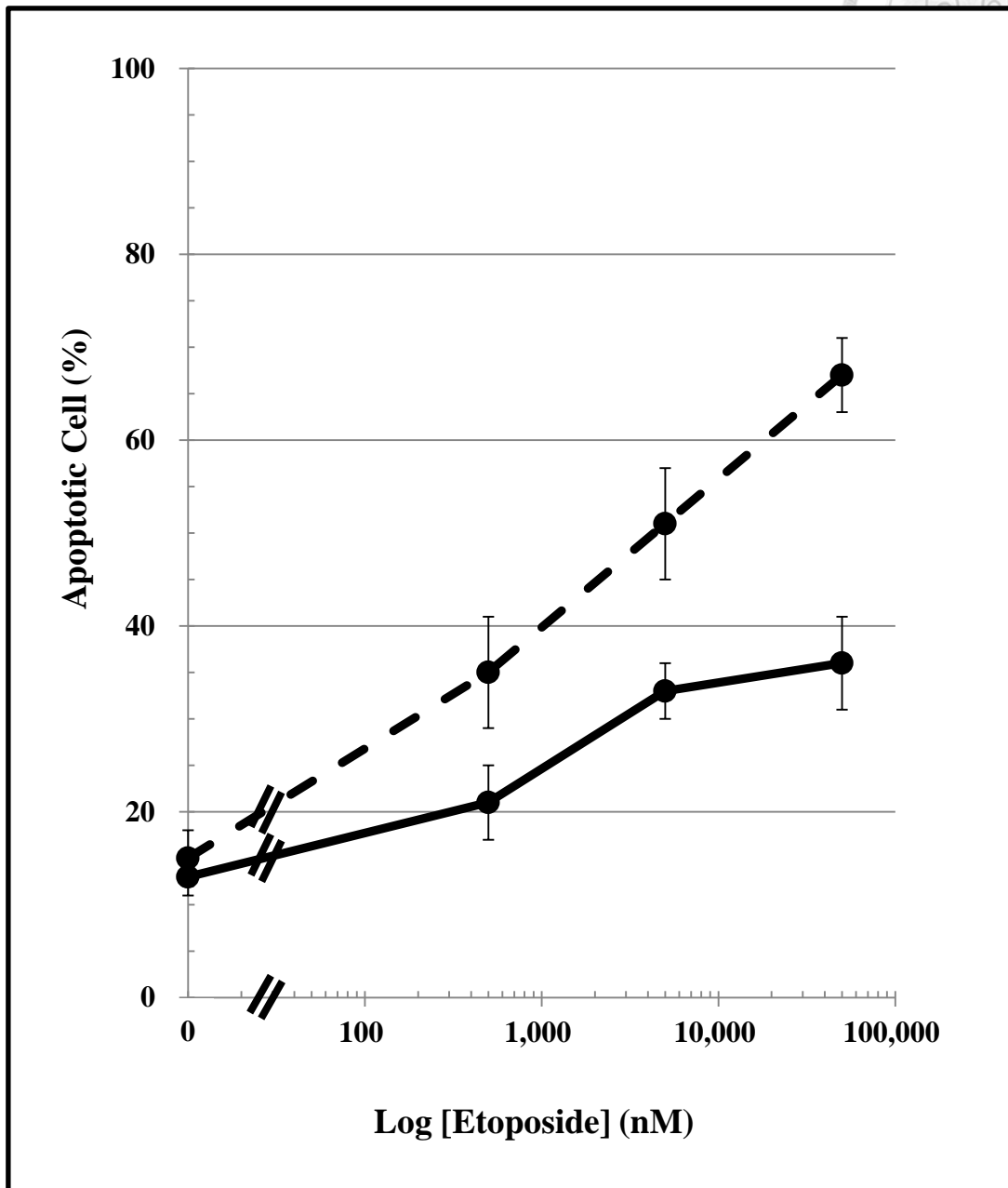
—— N cells, CD-133 positive pre-condition human neuroblastoma SH-SY5Y cells

－－－· D cells, Human neuroblastoma SH-SY5Y cell

4.2.2 Comparison of cell viability in D and N cells following etoposide treatment

Trypan blue exclusion method was used to measure the concentration-dependent response following a 24-hours treatment of N and D cells with etoposide (0 to 83 μM) (**Figure 6**). The present concentration-dependent cell viability curve was plotted on a semi-log paper which revealed a linear response curve in both D cells and N cells. The estimated IC_{50} values of etoposide in D cells and N cells were 700 nM and 19,000 nM, respectively. This result shows that N cells were less sensitive to etoposide than D cells by 27-fold. The cell mitochondria viability of both N and D cells has decreased measured by MTT method (**Figure 7**). But the decreased ratio of both cells was as the same level.
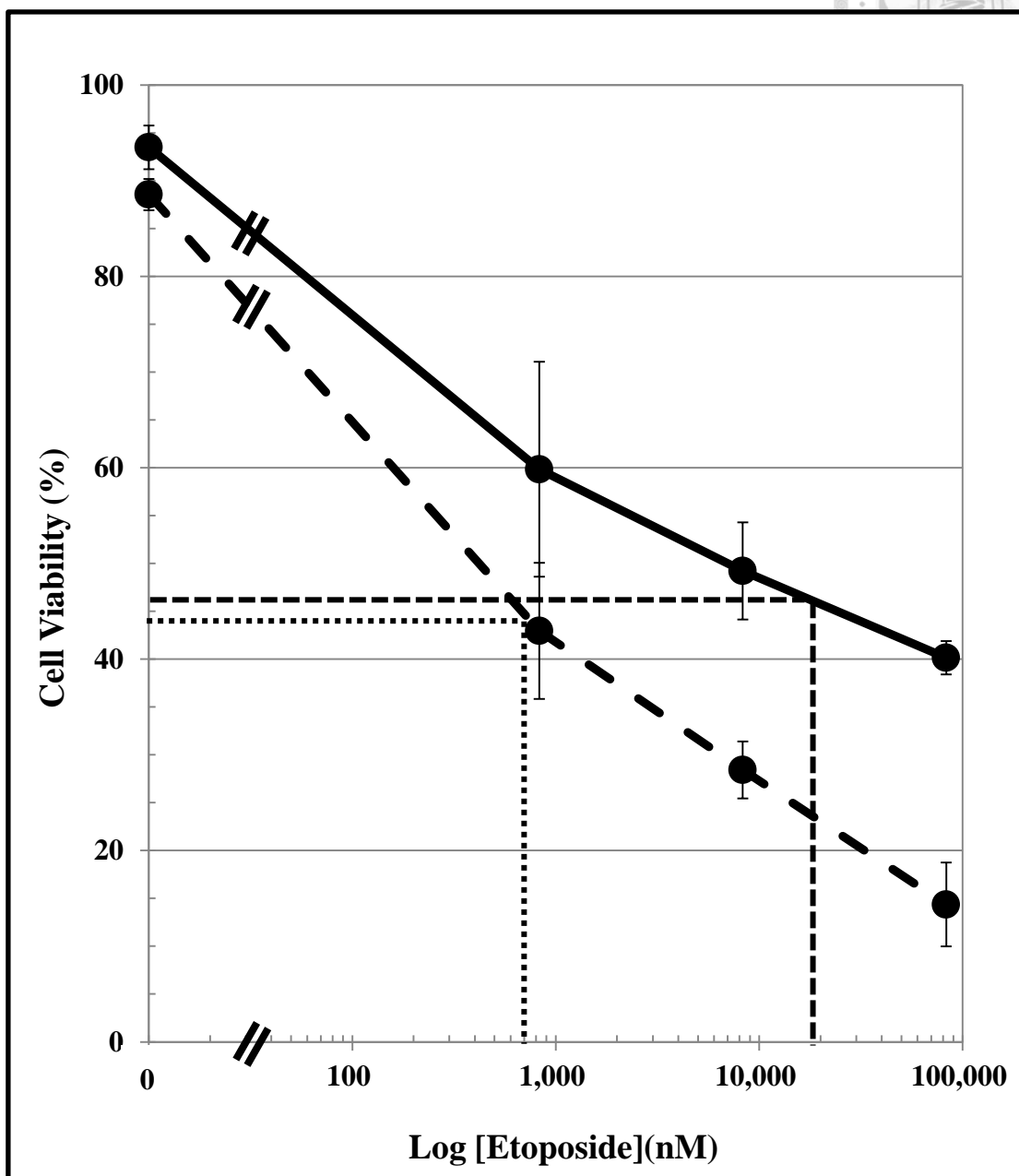
Figure 6. Comparison by Trypan blue exclusion method in D cells and N cells following Etoposide treatment (0 to 83 μM).

Cells were treated with etoposie (0 to 83 μM) for 24 hours and the cell viability was measured by the trypan blue exclusion method. (n = 6~8, mean ± SD)

IC$_{50}$ of D cells (•••••••) was estimated as 700 nM; IC$_{50}$ of N cells (▬ ▬ ▬·) was estimated as 1,900 nM. ▬▬▬▬ N cells, CD-133 positive pre-condition human neuroblastoma
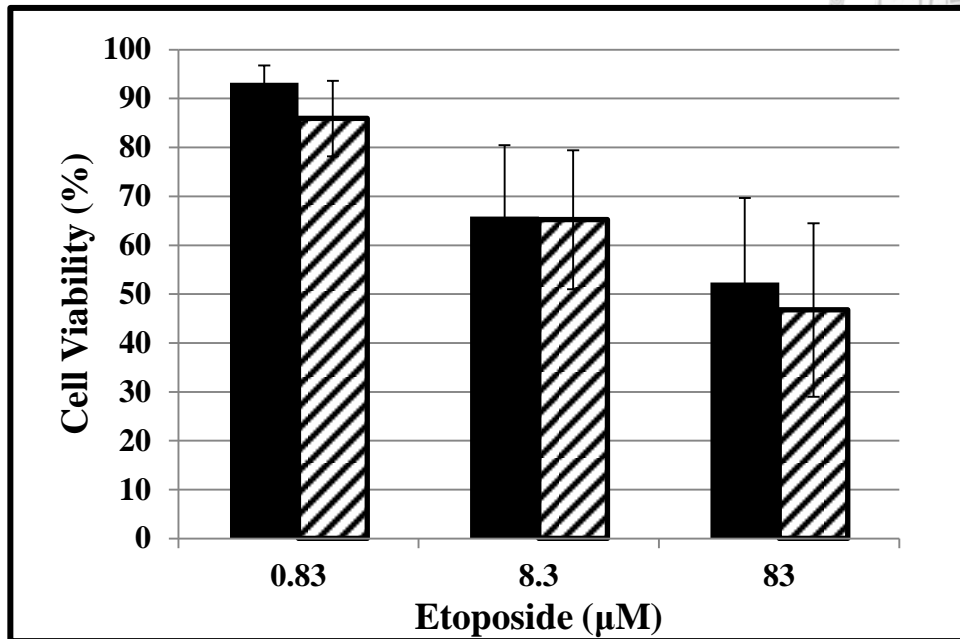
Figure 7. Effect of etoposide on cell viability, reflected by cell mitochondrial activity

was assayed by MTT method in D and N cells.

■ N cells, CD-133 positive pre-condition human SH-SY5Y cells

▨ D cells, Human neuroblastoma SH-SY5Y cells

(n = 12)

MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5diphenyltertrazolium bromide, was measured the

activity of cellular enzymes that reduce the tetrazolium dye to its insoluble formazen.

The color of this dye was changed from yellow to purple.

4.3 The effect of the inhibitor of redox cycling of Trx on etoposide-induced apoptosis in N cells

4.3.1 Effect of DNCB on etoposide-induced apoptosis in N cells

As the results that Trx expression was higher in N cells which were resistant to the etoposide-induced apoptosis and cell death; DNCB was used to block the redox cycling of Trx for understanding the role of endogenous Trx on the chemo-resistance in oncology.   To understand this, the etoposide treated N cells (0 to 83 μM) were co-treat with or without DNCB (50 μM).   Etoposide caused a concentration-dependent manner of apoptosis in both N and D cells (**Figure 8**).   Without DNCB co-treatment, etoposide caused a smaller apoptosis change in N cells than D cells by 1.8 times. Co-treatment of N cells with DNCB increased the sensitivity of N cells to etoposide-induced apoptosis which was similar to untreated D cells.

Without DNCB co-treatment (**Figure 9**), etoposide (83 μM) produced a greater apoptosis in D cells (67%) than N cells (35%).   But when the etoposide-treated N cells were co-treated with 50 μM of DNCB, the apoptotic cell population was increased significantly to 79% ($p < 0.05$), which was not statistically different from D cells with low Trx following treatment of D cells which was treated with etoposide.
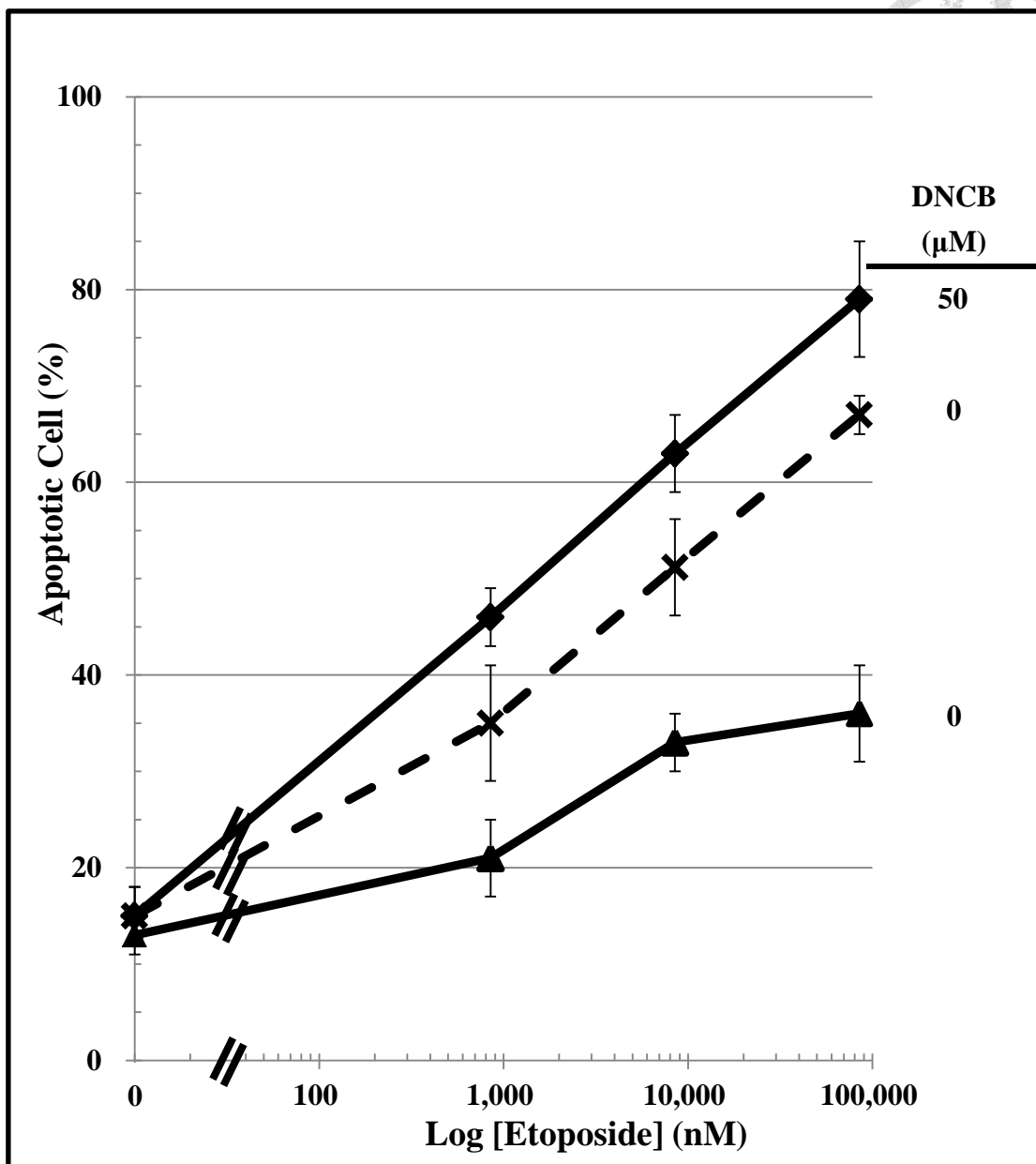
Figure 8. Effect of endogenous Trx redox cycling on the concentration-dependent apoptosis caused by etoposide in N cells.

N cells (━━━), CD-133 positive pre-conditioned human SH-SY5Y cells were co-treated with 0 or 50 μM DNCB and etoposide. Human neuroblastoma SH-SY5Y cells (D cells, ━ ━) were used as control. Each well was calculated by Hoechst 33258 nuclear staining for apoptotic changes in nuclei (i.e, nuclear condensation, fragmentation). DNCB was an inhibitor of Trx reductase which blocks the redox cycling of endogenous Trx in N cells. (n = 3, mean ± SD)
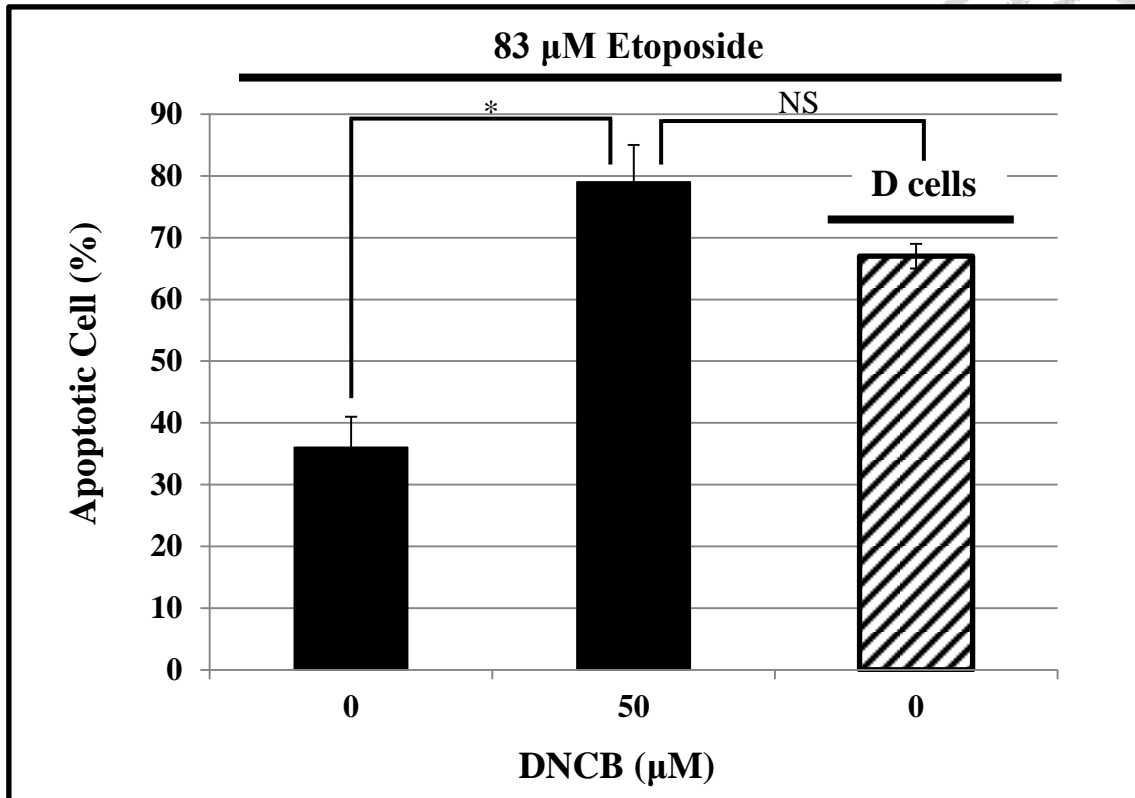
Figure 9. Statistical analysis of the effects of DNCB on the effect of etoposide in N cells.

Both D (▨) and N (■) cells were treated with etoposide (50 µM, 24 hours) to induce apoptosis measured by Hoechst 33258 nuclear staining method.

N cells were co-treated with DNCB (0 or 50 µM) for the inhibition of the redox cycling of Trx. (n = 3, mean ± SD); NS: not significant

*$p < 0.05$ N cells (0 vs. 50 µM DNCB)

$p > 0.05$ N cells (50 µM DNCB) vs. D cells (0 µM DNCB)

4.3.2 Effect of DNCB on Trx levels

The result of western blotting shows that the cellular Trx levels were not changed

in the DNCB (50 μM) treated N cells (**Figure 10**).   DNCB is an inhibitor of Trx

reductase, so that the redox cycling of Trx was blocked that led to the oxidative form of

Trx cannot be reduced back to the biol-active reduced form (Arner, Bjornstedt, and

Holmgren, 1995).

DNCB (µM)

| 0 | 5 | 10 | 50 |

12 kD- _____ Thioredoxin

43 kD- _____ Actin
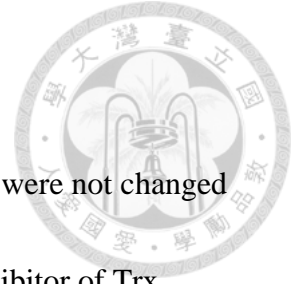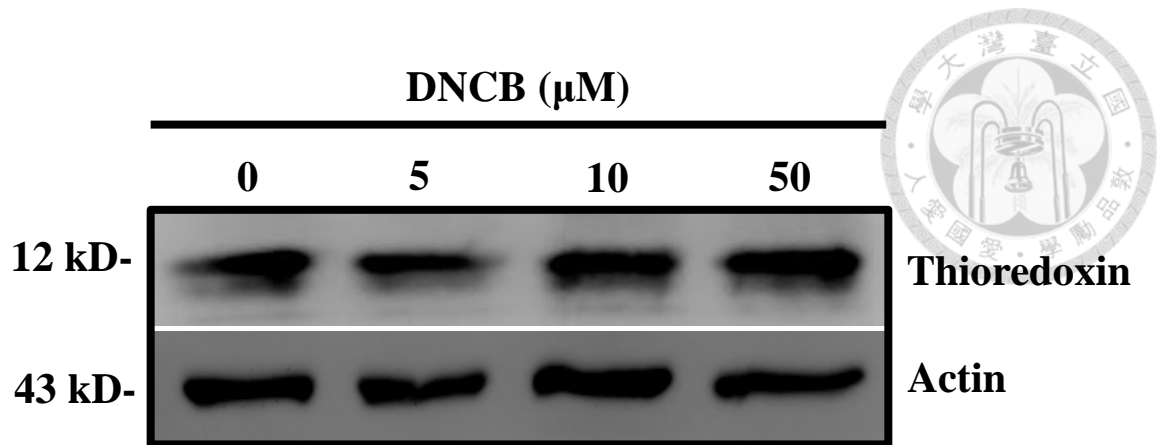
Figure 10. The expression of Trx in N cell with DNCB (0, 5, 10 and 50 µM) added by Western blotting.

CD-133 positive pre-condition human SH-SY5Y cells (N cells) following the treatment of etoposide (0 to 83µM) for 24 hours. Each land was loaded with 40µg of total protein extraction. The Trx expression was monitored by anti-human Trx polyclonal antibody, and the band was in 12 kD. Actin as loading control (43kD) was monitored by anti-human Actin monoclonal antibody. (n = 4)

DNCB, 1-Chloro-2,4-dinitrobenzene, which is an inhibitor of TrxR (Thioredoxin reductase).

4.4 The changes of etoposide-treated D cells

4.4.1 Trx level in etoposide-treated D cells

The Trx expression of human neuroblastoma SH-SY5Y cells (D cells) following

the treatment of etoposide (0 to 83 μM) for 24 hours was monitored by Western blotting

(**Figure 11**).　It showed that the expression were increased, and were reached highest

level at 8.3μM etoposide treated D cells by 3.8 fold.　At 83 μM etoposide treated D

cells, the expression of Trx was higher than untreated D cells by 2.3 fold.

4.4.2 Effect of etoposide on D cell morphology

Comparison of cell morphology on D and N cells has some differences between

two cells (**Figure 12**).　D cells were bigger and had much longer synapse than N cells.

But there has some changed after 83 μM etoposide treatment, D cells were much

smaller than before and its synapse were shorter even disappear.　Under Hoechst

33258 staining in nuclear, D cells nuclear changed from oval to round.　D cells looked

much more like N cells on morphology and nuclear shape after 83 μM etoposide

treatment.

(A)

(B)
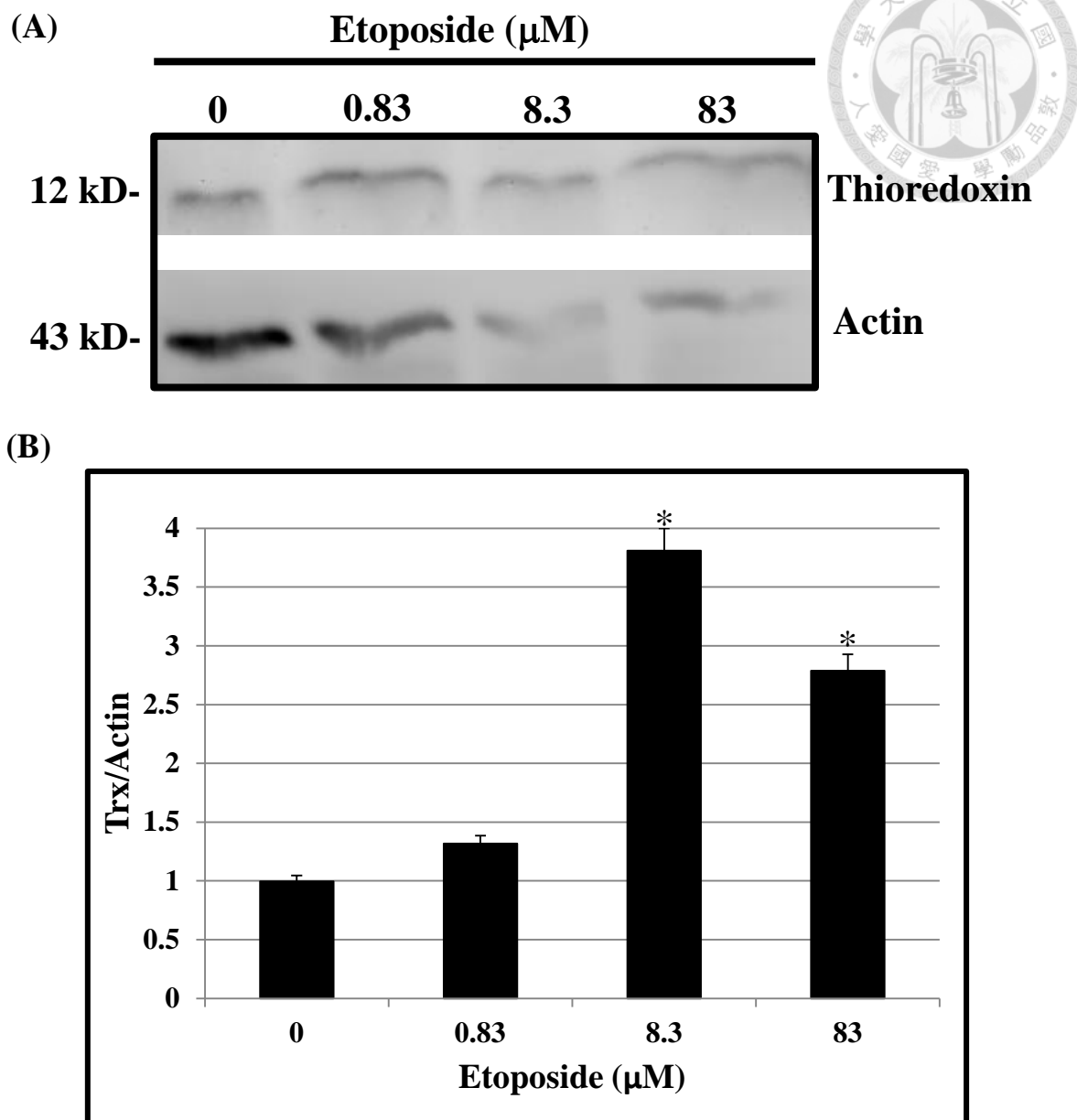
Figure 11. The expression of Trx in D cell with etoposide treatment (0 to 83 μM) by Western blotting.

Human neuroblastoma SH-SY5Y cells (D cells) treated with etoposide (0 to 83μM) for 24 hours. Each land was loaded with 40μg of total protein extraction and ran of 20% SDS-PAGE (A). The Trx expression was monitored by anti-human Trx polyclonal antibody, and the band was in 12 kD. Actin as loading control (43kD) was monitored by anti-human Actin monoclonal antibody. (B) The expression of Trx was measured by western blotting. (n = 3, mean ± SD) $*p < 0.05$

Etoposide (µM)	White Light	UV
0 (D cell)	20 µm	20 µm
83 (D cell)	20 µm	20 µm
0 (N cell)	20 µm	20 µm

Figure 12. The phenol type under white light and Hoehst 33258 nuclear staining of cell nuclear following etoposide treatment (0 or 83 µM) for 24 hours in N and D cells

Chapter 5: Discussion

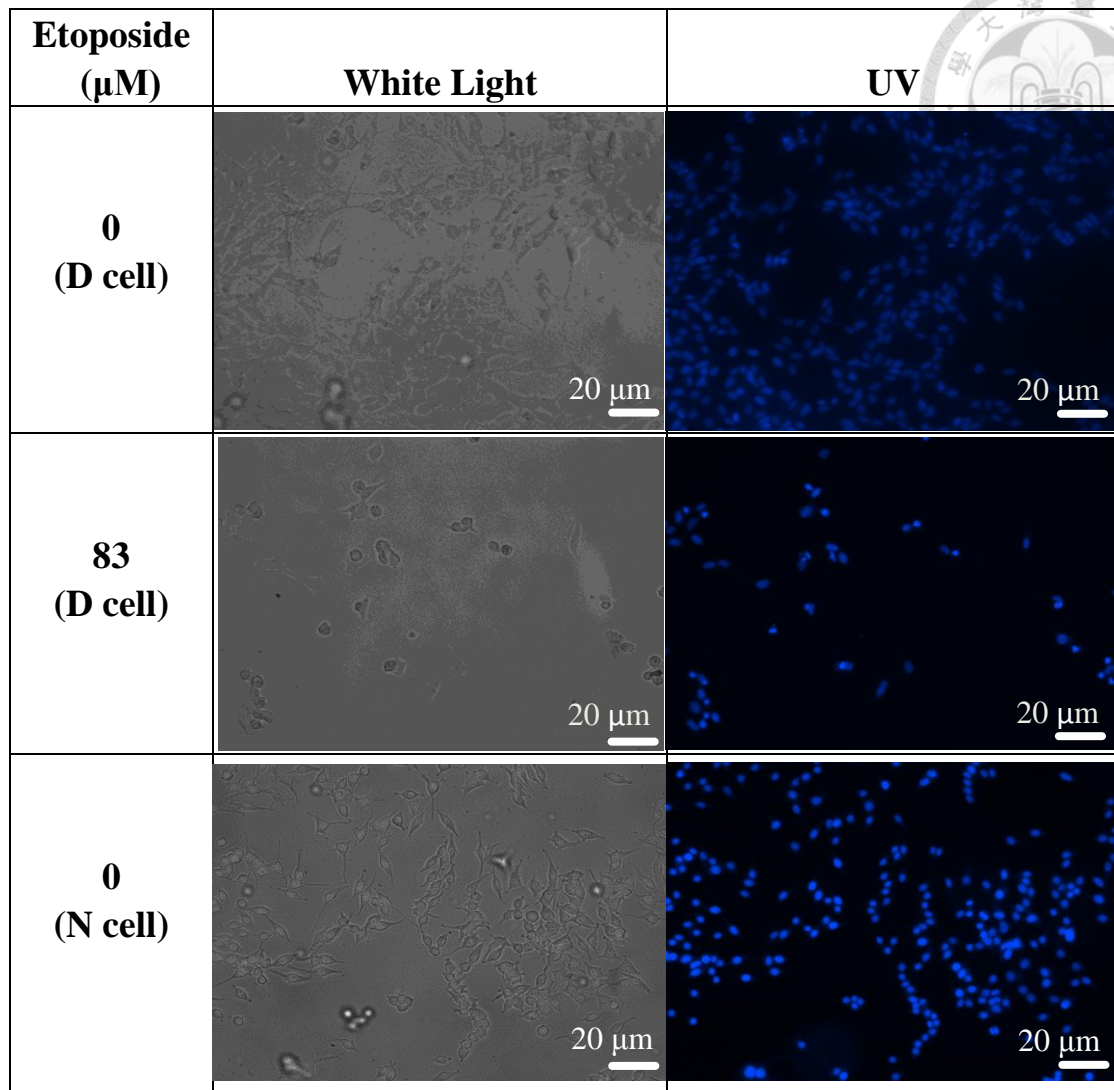The present results support a new research idea that increases the expression of Trx can prevent cancer cells from apoptotic cell death caused by etoposide (**Figure 13**). Etoposide form ternary complex with the topoisomerase II leading to DNA double strands break which cause cell apoptosis and related death as demonstrated in the present study that supports its indication (Gordaliza et al., 2004).   Andoh , Chock , and Chiueh, (2002) suggest that Trx prevents apoptosis by (i) increasing mitochondrial bcl2, (ii) decreasing pro-apoptotic cytochrome C and (iii) inhibition of pro-apoptoic caspase-3 and -9 enzymatic activities.   In fact, Trx prevents apoptosis and cell death caused by etoposide in N cells (**Figure 6**) through a redox cycling mechanism of Trx which was blocked by DNCB (**Figure 7**). The exact molecular event of anti-apoptotic mechanism of Trx reminds to elucidate further in the near future.

5.1 Role of Trx on etoposide-treated N cells.

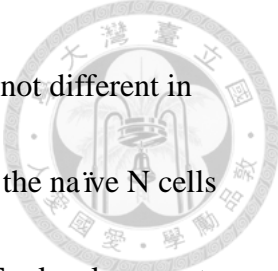Etoposide is one of the chemotherapy drugs for the treatment of multiple cancers via DNA double strands break (Geller et al., 1976) leading to apoptosis (**Figure 5**). Trx is expressed in cancer cells which might be responsible for poor treatment outcome (Andoh , Chock , and Chiueh, 2002).   Consistently, it has been reported that transfected Trx into cancer cells can increase cell survival (Yokomizo et al., 1995).

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The pre-conditioned human neuroblastoma SH-SY5Y cells contain high levels of

endogenous Trx (Andoh, Chock, and Chiueh , 2002).   The present results confirm

that Trx expression was higher in the preconditioned N cells than the wild type D cells

by 3 times (**Figure 3**).   Furthermore, Trx-enriched N cells were less sensitive than D

cells after etoposide-induced apoptotic change (**Figure 5**) and cell viability (**Figure 6**).

The results were in agreement with the prior report of transfecting Trx into cancer cells

leading to less apoptosis cell changes after chemotherapy (Geller et al., 1976).   On the

other hands, N cells were less sensitive than D cells by 27-fold by in cell death caused

by etoposide (**Figure 7**).   The etoposide-induced LD50 in N cells and D cells was

approximate 27-fold (N cells > D cells) which is consistent with a previous report on

apoptosis and cytotoxicity caused by a neurotoxin MPP+ in human neuroblastoma cells

with or without preconditioning (Andoh , Chock, and Chiueh , 2002).

DNCB is a Trx reductase inhibitor which can block the redox cycling of

endogenous Trx and thus related biological activities (Arner, Bornstedt, and Holmgren ,

1995).   Compared to low-Trx D cells, N cells had fewer cells under apoptosis if cells

were treated with 83 μM etoposide.   On the contrary, etoposide-treated N cells had

more cells under apoptosis if N cells were co-treated with 50 μM DNCB (**Figure 8**) to

block the redox cycling of Trx.   This result infers that Trx play a critical role in the

development of chemo-resistance following etoposide treatment.   The

concentration-dependent manner of etoposide-induced apoptosis was not different in

naïve D cells and N cells treated with 50 μM DNCB but much less in the naïve N cells

(**Figure 9**).   The Western blotting of Trx expression showed that Trx level was not

changed in DNCB-treated N cells (**Figure 10**), DNCB only affect the redox cycling of

Trx by blocking Trx reductase.   The oxidized form of Trx cannot be reduced to the

active reduced form (Elias and Holmgren, 2000).   These results are consistent with

previous reports that reductive form of Trx could play a critical functional role in cell

anti-oxidant, anti-apoptosis and anti-DNA damage (Andoh, Chock, and Chiueh, 2002).

The result of MTT method (**Figure 7**) was not as the same as the trypan blue

exclusion method, that there was no significant difference between N and D cells.

According to previous reports, MTT method measures the activity of mitochondria as

an index of the cell viability (Gerlier and Thomasset, 1986).   The negative result of

MTT shows that etoposide might have no effect on cell mitochondria activity.

Etoposide-induced cell death was not due to alteration of mitochondrial activity

reflecting by a negative MTT assay.   But etoposide may lead to DNA damage and

apoptosis resulting in cell death of cancer cells.

5.2 D cell may promote it cell survival by increased Trx expression

There are some kinds of cancers are resistant to chemotherapy. Neuroblastoma is one of them with poor survival outcome in the clinical after treatment and with high relapse rate (Marvis, 2010). The Trx level in the chemo-resistance cancer cells is higher than others that has been observed in neuroblastoma (Andoh, Chock, and Chiueh, 2002), but the Trx level after chemotherapy has not been report before. Here we have observed that Trx level was increased by 3.7 in etoposide-treated D cells (**Figure 11**) while morphology of D cells was altered to mimicking N cells in etoposide-treated D cells (**Figure 12**). The results shows that (i) cells may prevent from etoposide-induced DNA damage by increased its level and may have effect on the cell morphology that changed from D cell to N cell-like or (ii) etoposide may spare the Trx-enriched cells. On the other hands, actin has founded to be a pro-apoptosis signal in the extrinsic apoptosis cell death pathway (Desouza M, Gunning PW, and Stehn JR., 2012). Therefore, the increase of Trx in etoposide-treated D cells could be an error measured because of the decrease of actin. This error measured of Trx level in etoposide-treated D cells can be tested by other negative control protein, GAPDH, or measured RNA level by Real-time PCR. The other chemo-drug, vincristine (data not showed), also showed that N cells were less sensitive to the tubulin-target apoptosis than D cells, but

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Trx level has not changed after treatment with vincristine (0 to 2.5 μM). Cancer cells

may elevate Trx levels to protect itself from DNA-damage stress and apoptosis evoked

by etoposide but not vincristine-induced tubulin stress.
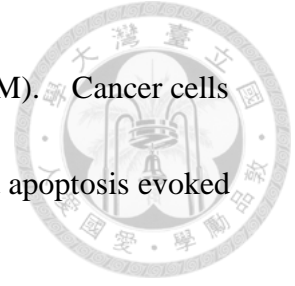
Figure 13. Trx Pathway Blocks etoposid-induced DNA Breaks
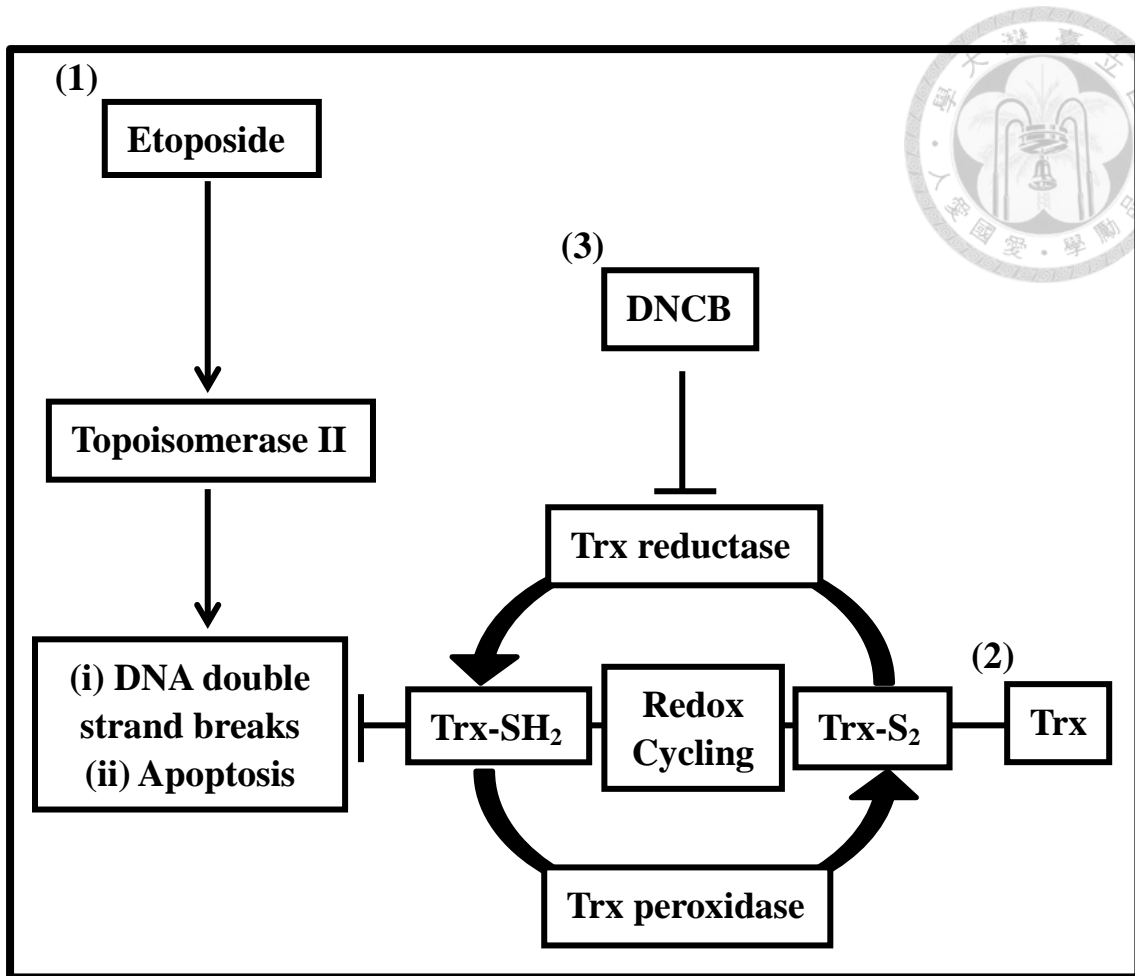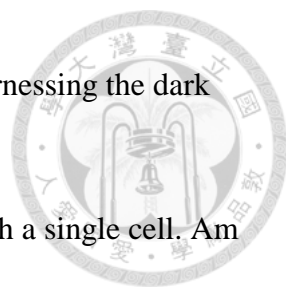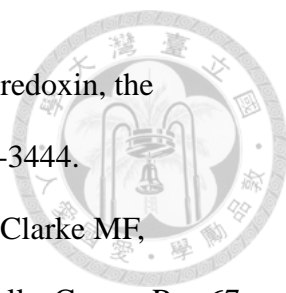
(1) Etoposide forms a ternary complex with DNA and Topoisomerase II lead to DNA

double strand breaks.

(2) Trx may inhibit the DNA break and apoptosis caused by etoposide.

(3) DNCB is a Trx reductase inhibitor, it blocks the redox cycling of Trx so that the

reduced form of Trx cannot executive it function to inhibit the DNA break and

apoptosis caused by etoposide.

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問與答

論文口試摘要

本論文口試 2013 年 6 月 24 日上午 10 點於生化科技所 509 室舉行，承蒙下列五位老師：莊榮輝教授、關狀卿教授 (本論文共同指導教授)、許秀蘊教授、楊健志副教授以及黃楓婷助理教授審核。口試摘要匯集書面補充回答後，依照提問順序加以記錄及整理。

許秀蘊老師：

1. Wild type chemoresistance cell line 是否在 hypoxia 狀態下從 D cell 篩選出來?Trx 及 CD133 表現?

回答: D cell 為 wild type 也就是一般 commercial 的 cell line，其 Trx 表現量較低或是沒有表現; N cell 則是由 D cell 經過許多 stress 篩選出來的 cell line，其會表現較高量的 Trx。CD133 則是細胞表面的抗原，現今則是作為腦神經 CSC 的特殊表面抗原指標；經本實驗室鄔承佑學長的實驗發現，D cell 不會表現 CD133 而 N cell 則會表現。

2. Etoposide 濃度參考為何?

回答: Etoposide 濃度為參考一篇同樣以 neuroblastoma 作為研究材料之 paper (Ruth et al.,2002)。

3. IC_{50} 的單位為何用 nM?

回答: 起初是以 μM 做 semi-log 的圖，但是因為後來發現要比較精準計算 IC_{50} 的話，以 nM 較好做圖計算，因此才以 nM 作圖呈現。

楊健志老師：

1. 利用 Trx/Actin ratio 做比較是否適當?

回答: 由於沒看過 paper 有做過，在加藥後進行 Western blotting 比較蛋白質量的差異，因此這部分算是新的嘗試。由於在高濃度作用下 (**Figure 11**)，細胞大部分都死亡，所以一次需要收集多盤的細胞，定量上也有困難。所以 **Figure 11** 中以 Trx/Actin ratio 作為定量的結果，結果可能是因為 (i) Trx

表現量確實有改變或是 (ii) 因為 Actin 為 apoptosis pathway 上游之 signal，所以 Actin 的量下降了導致以 Trx/Actin ratio 上升。之後可嘗試改變 internal control 或是利用 real-time PCR 定 RNA 量是否改變來做確定。

2. Hoechst 33258 判定細胞死活的標準?

回答: 以 **Figure 4** 中表示，以細胞核碎裂、細胞核萎縮、細胞核大小改變座位細胞凋亡現象的依據。

3. Thioredoxin reductase inhibitor 對於 normal cell 也有作用，如何解決?

回答: 在與闕老師討論時，我也有想到這問題。因此在想是否可與現今發展之 CSC marker 做結合，即像是 DNCB 與 CSC marker 之 Antibody 結合，使藥物作用能有專一性。

4. 如何確保永久繼代 N cell?

回答: 有使用低濃度FBS作篩選，作為給N cell的stress。也有嘗試過將N cell 養回 D cell 的 medium 中，也不會產生變異，具有穩定性。

黃楓婷老師:

1. Etoposide concentration 8.3~83 μM 差距很大，是否恰當?

回答: 一開始我是以 paper 中的濃度 (8.3 μM)去做初步測試，並以上下兩倍差的作一曲線，結果顯示不是很理想，看不太出藥物作用的差異，因此最後在老師建議下，把間距拉為 10 被之後才看得出藥物對細胞的影響，也才做得出 semi-log 的圖 (**Figure 5**)。

2. 現在使用的濃度是否符合臨床藥物的濃度?

回答: 之前和老師有換算過，是符合的。

3. DNCB 對於 Trx 是否有專一性?

回答: DNCB 是作用於 Trx reductase 的抑制劑，對於 Trx reductase 有專一性。

莊榮輝老師:

1. DNCB 對於人體是否有毒性?

回答: DNCB 目前未使用在臨床上，所以不知對人體毒性為何，但是臨床上有其他的 Trx reductase 抑制劑在使用。在細胞加藥的過程中觀察，單一只加入 DNCB 對於細胞影響不是很大，細胞凋亡和 control 比沒有差很多，所以以此作為判斷的話，DNCB 對於人體毒性可能還好。

2. 在加藥實驗的部分，是否可能是 medium 裡的 Trx 作用?你是否有測過 medium 裡的 Trx?

回答: 目前沒有做過，但是的確有可能，因為 Trx 已經被證實會被分泌到細胞外，作為 cell comunication 的 signal，未來可以嘗試使用 medium 作 Western blotting 來做為參考。

3. Trx 與 CSC 之間的關係是必要的嗎?Trx 在 N cell 一直表現之原因?

回答: 目前還沒有做到這 DNA 的部分，但的確在一般細胞沒有受到 stress 是不表現 Trx，因此 N cell 會穩定表現 Trx 的確是很特殊的狀況，由於 N cell 與 CSC 類似，所以在經過 stress 後可能改變了 Trx 的基因表現，這也可以是未來的研究方向。

關狀卿老師:

1. Drug-resistant patient 投 DNCB 藥物?你認為可否做輔助治療?

回答: 我認為是可以的，以 **Figure 8** 的結果顯示，在抑制 Trx 後可有效地殺死 N cell，也就是可以使難以治療並且表現較高 Trx 之癌症細胞。

2. 和 2002 年 Andoh 的研究發現比較，有何新的發現?

回答: 此次主要發現抑制 Trx 後，可使 N cell 有效的被殺死外 (**Figure 8**)，另外還有發現 D cell 在 etoposide 作用下產生的變異，像是 Trx 表現量的改變 (**Figure 11**)，以及 cell morphology 改變的現象 (**Figure 12**)