บทบาทของไตรเอริลมีเทน-34 ต่อช่องไอออนโพแทสเซียมชนิดโพแทสเซียมแคลเซียม 3.1 ต่อเซลล์ไลน์ไตแมวที่ถูกเหนี่ยวนำด้วยด๊อกโซรูบิซิน

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THE ROLE OF TRIARYLMETHANE-34 ON POTASSIUM CHANNEL KCA3.1 IN DOXORUBICIN-INDUCED FELINE KIDNEY CELL LINE

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ปราลี นันทรักษ์ชัยกุล : บทบาทของไตรเอริลมีเทน-34 ต่อช่องไอออนโพแทสเซียมชนิดโพแทสเซียมแคลเซียม 3.1 ต่อเซลล์ไลน์ไตแมวที่ถูกเหนี่ยวนำด้วยด๊อกโซรูบิซิน (THE ROLE OF TRIARYLMETHANE-34 ON POTASSIUM CHANNEL KCA3.1 IN DOXORUBICIN-INDUCED FELINE KIDNEY CELL LINE) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. สพ.ญ. ดร.รสมา ภู่สุนทรธรรม, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. ดร.ฉัตรศรี เดชะปัญญา, 92 หน้า.

การศึกษาผลของการยับยั้งช่องไอออนโพแทสเซียมชนิดโพแทสเซียมแคลเซียม 3.1 (ไตรเอริลมีเทน-34 triarylmethane-34 (TRAM-34)) ต่อเซลล์ไลน์ไตแมวที่ถูกเหนี่ยวนำให้เกิดไตวายด้วยด๊อกโซรูบิซิน doxorubicin (DOX) การศึกษาแบ่งออกเป็นสองส่วน ส่วนที่หนึ่งศึกษาหาขนาดต่ำสุดของ TRAM-34 ที่ไม่ก่อให้เกิดความเป็นพิษต่อเซลล์ไลน์ไตแมว โดย cytotoxicity assay ส่วนที่สองศึกษา TRAM-34 ในขนาดที่เหมาะสมถูกบ่มในเซลล์เพาะเลี้ยงไตแมวที่ถูกโน้มนำให้เป็นพิษด้วย DOX ก่อนการรักษาเป็นเวลา 24 ชั่วโมง หรือ 30 นาที และหลังการรักษา 24 ชั่วโมง โดยทำ cytotoxicity assay, apoptosis assay, necrosis assay และตรวจหาการแสดงออกของโปรตีนของช่องไอออนโพแทสเซียมแคลเซียม 3.1 ในเซลล์ไลน์ไตแมว ผลการศึกษาพบว่าอัตราการรอดของเซลล์ไลน์ไตแมวในกลุ่มที่ให้ TRAM-34 ที่ขนาด 0.1 ถึง 100 ไมโครโมลาร์ (μM) ไม่พบความแตกต่างอย่างมีนัยสำคัญทางสถิติเมื่อเทียบกับกลุ่มควบคุมใน 24 ชั่วโมง แต่พบอัตราการรอดของเซลล์ไลน์ไตแมวลดลงอย่างมีนัยสำคัญทางสถิติเมื่อใช้ TRAM-34 ที่ขนาด 100 μM ใน 48 ชั่วโมง (p<0.05) และขนาดยาต่ำสุดที่ไม่ทำให้เป็นพิษต่อเซลล์ไลน์ไตแมว คือ 100 μM ของ TRAM-34 ใน 24 ชั่วโมง, 50 μM ของ TRAM-34 ใน 48 ชั่วโมง และ 25 μM ของ TRAM-34 ใน 96 ชั่วโมง ตามลำดับ นอกจากนี้ยังพบว่าเซลล์ไลน์ไตแมวที่ให้ TRAM-34 ขนาด 0.1 ถึง 1 μM ที่ 24 ชั่วโมง ก่อนเหนี่ยวนำด้วย DOX มีเปอร์เซ็นต์การรอดชีวิตของเซลล์สูงกว่า และมีเปอร์เซ็นต์การตายแบบอะพอพโทซิส (apoptosis) น้อยกว่ากลุ่มควบคุม DOX อย่างมีนัยสำคัญทางสถิติ (p<0.05) แต่พบว่าไม่มีความแตกต่างอย่างมีนัยสำคัญทางสถิติของเปอร์เซ็นต์การตายแบบเนโครซิส (necrosis) เมื่อเทียบกับกลุ่มควบคุม DOX นอกจากนี้การให้ TRAM-34 ขนาด 0.1 μM ในเซลล์ไลน์ไตแมวก่อน 24 ชั่วโมงที่จะเหนี่ยวนำด้วย DOX พบว่ามีการลดลงของการแสดงออกของโปรตีนของช่องไอออนโพแทสเซียมแคลเซียม 3.1 เมื่อเปรียบเทียบกับกลุ่มควบคุม DOX อย่างมีนัยสำคัญทางสถิติ (p<0.05) ดังนั้น TRAM-34 สามารถป้องกันเซลล์ไลน์ไตแมวจากการโน้มนำให้เกิดไตวายด้วย DOX โดยยับยั้งช่องไอออนโพแทสเซียมแคลเซียม 3.1 และอาจสามารถนำมาใช้ในการรักษาโรคไตเรื้อรังของแมวได้ในอนาคต

ภาควิชา	อายุรศาสตร์	ลายมือชื่อนิสิต	.....
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KEYWORDS: CATS / DOXORUBICIN / FELINE KIDNEY CELL LINE / KCA3.1 CHANNEL INHIBITOR

PARALEE NANTARAKCHAIKUL: THE ROLE OF TRIARYLMETHANE-34 ON POTASSIUM CHANNEL KCA3.1 IN DOXORUBICIN-INDUCED FELINE KIDNEY CELL LINE. ADVISOR: ASSOC. PROF. ROSAMA PUSOONTHORNTHUM, D.V.M., M.Sc., Ph.D., D.T.B.V.M., CO-ADVISOR: ASSOC. PROF. CHATSRI DEACHAPUNYA, B.Sc., M.Sc., Ph.D., 92 pp.

Study involved with potassium channel KCa3.1 blocker (triarylmethane-34; TRAM-34) in doxorubicin (DOX)-induced feline kidney cells was performed. The study was divided into two parts. In part I, subtoxic dose of TRAM-34 in feline kidney cell lines was studied to determine the cytotoxicity of TRAM-34 in feline kidney cells by cytotoxicity assay. In part II, feline kidney cell lines were incubated with appropriate dose and time of TRAM-34 pretreatment for 24 h or 30 min and post-treatment for 24 h condition in DOX-induced cell toxicity. Cytotoxicity assay, apoptosis and necrosis assay and KCa3.1 protein expression were measured. The cytotoxicity results indicated no significantly differences in cell viability between cells treated with TRAM-34 at 0.1 to 100 μM concentration and negative control in 24 h but a significant reduction of cell surviving at 100 μM concentration of TRAM-34 in 48 h (p<0.05). We found subtoxic dose of TRAM-34 in feline kidney cells lines at the concentration 100 μM in 24 h, 50 μM in 48 h and 25 μM in 96 h when compared with the negative control. Pretreatment with TRAM-34 at 0.1 to 1 μM concentrations for 24 h had significantly higher percentages of cell viability (p<0.05) and significantly lower percentages of apoptotic cells respect to the total than DOX-treated control (p<0.05) but was not significantly different in percentages of necrosis cells than DOX-treated control. Moreover, Pretreatment with TRAM-34 at the 0.1 μM concentrations for 24 h had significantly decreased KCa3.1 protein expression when compared with DOX-treated control (p<0.05). Therefore, these findings suggested that TRAM-34 can protect feline kidney cells line from DOX-induced toxicity by inhibiting KCa3.1 channel. KCa3.1 channel blocker may be used as one of the potential therapeutic treatment for cats with naturally-occurring chronic kidney disease in the future.

Department:	Veterinary Medicine	Student's Signature	.....
Field of Study:	Veterinary Medicine	Advisor's Signature	.....
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LIST OF ABBREVIATIONS

APS	Ammonium persulfate
ATCC	American type culture collection
β	Beta
CaM	Calmodulin
CCL20	Chemokine ligand 20
CKD	Chronic kidney disease
CRFK	Crandell-Reese feline kidney
DOX	Doxorubicin
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfide
EDTA	Ethylene diamine tetraacetic acid
ERK1/2	Extracellular signal-regulated kinase ½
FBS	Fetal bovine serum
g	Grams
GFR	Glomerular filtration rate
h	Hours
HRP	Horseraddish peroxidase
IC50	Half maximum inhibitory concentration
K	Potassium
KCa	Potassium calcium
KCa3.1	Potassium calcium channel 3.1
kDa	Kilodalton
Kg	Kilograms
l	Liter
M	Molar

ml	Milliliter
µg	Microgram
µl	Microliter
µM	Micromolar
MCP-1	Monocyte chemoattractant protein-1
min	Minute
mM	Millimolar
nm	Nanometer
nM	Nanomolar
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PI	Propidium Iodide
PVDF	polyvinylidene fluoride
SDMA	Symmetric dimethylarginine
SDS	Sodium dodecyl sulfate
SEM	Standard error of mean
TBS-T	Tris-buffered saline-Tween20
TEMED	Tetramethylethylenediamine
TGF-β1	Transforming growth factor-beta1
Thr	Threonine
TRAM-34	Triarylmethane-34
U	Unit
UUO	Unilateral ureteral obstruction
Val	Valine
v/v	Volume/volume
w/v	Weight/volume

CHAPTER I
INTRODUCTION

Chronic kidney disease (CKD) is an important disease in geriatric cats. The prevalence of CKD in cats is 1.6–20% depend on the studies (Boyd et al., 2008). At present, the prevalence is increasing in geriatric cats (Reynolds and Lefebvre, 2013). In the USA, 1.9% of cat in veterinary practices was found with CKD (Lund et al., 1999). In Australia, cats with CKD presented 20% of the unhealthy cats in the veterinary hospitals (Watson, 2001). In Thailand, 6 CKD cats per 1000 cats were cats with CKD visited the veterinary hospitals (Pusoonthornthum et al., 2010). In the USA, 53% of CKD cats were over 7 years old (DiBartola et al., 1987) with the average age of 12.6 years (Elliott and Barber, 1998). In Thailand, the average age of cats with CKD were 6 years old. The most common breed for CKD is Siamese and domestic short haired (Pusoonthornthum et al., 2010). This disease is a leading cause of death and illness in domestic cats, in Thailand which causes much concern and economic loss to the Thai geriatric cats owners. Cats with CKD cannot be cured and medical management such as symptomatic and supportive treatment remains the standard of care (Polzin, 2010; Habenicht et al., 2013).

The pathophysiology of the CKD is complex and often associated with many factors (Harris and Neilson, 2006; Nangaku and Fujita, 2008). There are many potential etiology of CKD in cats but the most common cause is chronic tubulointerstitial nephritis (DiBartola et al., 1987; Grauer, 2009). The most common kidney pathological lesion of feline CKD is tubulointerstitial fibrosis (Lawler et al., 2006). Renal tubulointerstitial fibrosis is accepted as the final pathway for feline CKD (Reynolds and Lefebvre, 2013; Lawson et al., 2015). Feline CKD was involved with many mediators in renal fibrosis (Lawson et al., 2015). Many pro-fibrotic mediators secreted by cells involved in the inflammation of feline kidney such as transforming growth factor-β1 (TGF-β1) that initiates renal fibrogenesis (Arata et al., 2005; Bobkova et al., 2006). The urinary TGF-β1 concentration has been found to increase in cats with CKD (Arata et al., 2005; Habenicht et al., 2013). In addition, studies had been indicated that cats with

CKD had significantly higher oxidative stress than the clinically normal cats (Keegan and Webb, 2010; Piyarungsri et al., 2014).

Doxorubicin (DOX) is a substance known to be toxic to the kidney cells. In renal tubular cell, doxorubicin-induced cell apoptosis has been reported in both in vivo and vitro studies. Renal tubular cell apoptosis was proposed mechanism of CKD and plays a role in the pathophysiology of renal disease in human (Hauser and Oberbauer, 2002). Several experimental CKD models were induced by the intravenous injection of DOX in rats (Yoneko et al., 2007). DOX can induce apoptosis in human renal cell line (Chaotham et al., 2013). In addition, the main factor of doxorubicin-induced nephrotoxicity was the oxidative stress. DOX can activate O_2 molecule to create reactive oxygen species (ROS) (Muller et al., 1997). Increasing of oxidative stress and eNOS gene and protein expression was found in DOX-induced feline kidney cell line (Piyarungsri et al., 2014).

At present, there is no specific treatment to slow down the progression of renal fibrosis in human or cats. Therefore, researcher has focus on identifying factors which drive the progression of renal fibrosis and to identify potential therapeutic targets (Lawson et al., 2015). Previous studies has demonstrated the role of ion channels with kidney disease. Potassium channels is one channel that has been reported to be involved in kidney disease. Recently, the studies investigate the intermediate-conductance $Ca2^{+}$-activated K^{+} channel KCa3.1 (KCa3.1) and reported that it play a role in renal fibrosis. (Grgic et al., 2009a; Mene and Pirozzi, 2010; Huang et al., 2013; Mene and Pirozzi, 2013; Huang et al., 2014d; Huang et al., 2015). KCa3.1 channel is called IKCa1, IK1, SK4 and KCNN4 (Wulff and Castle, 2010; Chen et al., 2016). These channel is present in various cells in renal fibrotic process, including proximal tubular cells, fibroblasts, T-lymphocytes, macrophages and endothelial cells (Huang et al., 2014c). KCa3.1 channel is one of target for kidney disorders such as polycytic kidney disease, renal fibrosis, diabetic nephropathy, kidney allograft rejection and chronic kidney disease (Bertuccio and Devor, 2015). KCa3.1 plays the role in renal fibroblast proliferation and fibrogenesis (Huang et al., 2014d) and induction of KCa3.1 expression linked to apoptosis in human kidney cells (Chen et al., 2016). Insufficiency or Inhibition of KCa3.1 suppressed the development of renal fibrosis in mice (Grgic et al., 2009a)

and reduced expression of TGF-β1 induced monocyte chemoattractant protein 1 (MCP-1) in human proximal tubular cells (Huang et al., 2014a). Moreover, KCa3.1 is involved in the tubulointerstitial fibrosis in diabetic nephropathy (Huang et al., 2013; Huang et al., 2014d). In mouse, blockade of KCa3.1 reduced TGF-β1 level in diabetic nephropathy (Huang et al., 2013). In addition, renal fibroblasts of diabetic mouse kidneys exposed to TGF-β1 had reduction of fibronectin, type I collagen, vimentin, α-smooth muscle actin and fibroblast-specific protein-1 by KCa3.1 channel inhibitor (Huang et al., 2014d). Therefore, blockade of KCa3.1 can reduce the fibrotic and inflammatory processes in the kidney (Bertuccio and Devor, 2015). KCa3.1 inhibitors such as TRAM-34 may be a potential renal therapeutics for CKD (Bertuccio and Devor, 2015).

Up to present, the etiology of CKD in cats is unknown and whether potassium channel KCa3.1 is involve in the process of renal injury in cats remain to be investigated. The present study is designed to study the role of KCa3.1 channel inhibitor (triarylmethane-34; TRAM-34) in doxorubicin-induced feline kidney cells. Further information of KCa3.1 role in CKD cats may lead us to a better understanding about pathophysiology of feline CKD and may be useful in finding a new therapeutic approach for this important disease in the future.

Objectives of the study

To investigate the role of triarylmethane-34 (TRAM-34) on potassium channel KCa3.1 in doxorubicin-induced feline kidney cell line.

Hypothesis

There are differences in cell viability, apoptosis and KCa3.1 expression among controls, triarylmethane-34 (TRAM-34) treated and untreated of DOX-induced feline kidney cell line.

Keywords (Thai): แมว, ด๊อกโซรูบิซิน, เซลล์ไลน์ไตแมว, ตัวยับยั้งช่องไอออนโพแทสเซียมแคลเซียม 3.1

Keywords (English): cats, doxorubicin, feline kidney cell line, KCa3.1 channel inhibitor

Advantages of Study

KCa3.1 channel blocker can be used as a therapeutic treatment for cats with naturally-occurring chronic kidney diseases in the future.

CHAPTER II
LITERATURE REVIEW

2.1. Feline chronic kidney disease

Chronic kidney disease (CKD) is a progressive loss of kidney function and/or structure that has been present for more than 3 months (Polzin, 2010). Chronic kidney disease (CKD) is an important problem of ageing cats. The prevalence of CKD in cats is 1.6–20% and is higher than in dogs (0.5-7%) (Boyd et al., 2008). The prevalence of CKD appears to be increasing (Reynolds and Lefebvre, 2013). This disease is a leading cause of sickness and death in domestic cats. Cats with CKD cannot be cured and medical management remains the standard of care (Polzin, 2010; Habenicht et al., 2013). The important treatment for feline CKD is supportive and symptomatic treatment (Scherk, 2012; Korman and White, 2013).

The causes of CKD are heterogeneous and most often not identified. It can be congenital and acquired (Reynolds and Lefebvre, 2013). There are many potential etiology of CKD in cats but the most common cause is tubulointerstitial nephritis (Grauer, 2009). Other potential causes of feline CKD are found such as glomerulonephritis from immunologic disorders, upper urinary tract due to the uroliths, pyelonephritis from Infectious causes, amyloidosis, polycystic kidneys and renal dysplasia in familial renal disease, renal neoplasm and urinary outflow obstruction (Grauer, 2009; Chew et al., 2011; Reynolds and Lefebvre, 2013).

There are several risk factors for CKD in cats including ageing, breed, gender, systemic hypertension, cardiovascular disease, primary hyperaldosteronism and urinary tract infection (Reynolds and Lefebvre, 2013). Aging are the important risk. The prevalence of CKD rises with age and at least 15-30% is found in cats were older than 15 years old (Lulich et al., 1992). In the USA, the average age of feline patients with CKD was 7.4 years old (Polzin et al., 1989). In Thailand, the average age of cats with CKD was 6 years old (Pusoonthornthum et al., 2010) and 11.17±0.86 years (Jaimun et al., 2012). In addition, the average age of CKD cats with metabolic acidosis were 9.3 years old and 77% of cat with CKD were over 7 years old (Pusoonthornthum et al., 2012). Cat breed is one of the risk factors to developing CKD with high prevalence of

CKD in Abyssinian, Persian, Maine coon, Siamese, Burmese cats and Russian blue (Lulich et al., 1992). In Thailand, Siamese and Siamese-mixed breed were commonly found in CKD cats (Pusoonthornthum et al., 2010). Gender is other risk factors of CKD. Female cats incline to have CKD lower than male cats. Systemic hypertension, primary hyperaldosteronism, cardiovascular disease and urinary tract infections were mediate the development of CKD (Reynolds and Lefebvre, 2013). Recently, one study showed association between feline paramyxovirus (FPaV) infections and feline CKD (Sieg et al., 2015). In Thailand, Male, tap water and an outdoor lifestyle increased the risk for CKD in cats with naturally occurring CKD cats (Piyarungsri and Pusoonthornthum, 2017). In addition, one study in UK found development of feline CKD in cats associations between both severity of dental disease and vaccination frequency (Finch et al., 2016).

The common clinical signs of cats with CKD are inappetence, lethargy, weight loss, polyuria, polydipsia, halitosis, nausea and vomiting. Cats with CKD are dehydration, poor body condition, unkempt hair coat, small and irregular kidneys on kidney palpation and pale mucous membrane on physical examination (Paepe and Daminet, 2013; Polzin, 2013).

There are several diagnostic technique for CKD in cats. Feline CKD is diagnosed based on signalments, history and clinical signs. Blood test especially serum creatinine and urine analysis are important diagnostic tools. Symmetric dimethylarginine (SDMA) is a new blood biomarker in cats for detection of CKD before diagnosis by measurement of serum creatinine (Hall et al., 2014). Abnormal appearances of kidneys are diagnosed by medical imaging technique such as radiography and ultrasound (Paepe and Daminet, 2013). Moreover, Renal biopsy and GFR measuring can be used as diagnostic tools but there are potential significant risk and not as a routinely performed (Scherk, 2012; Paepe and Daminet, 2013). Lately, Urinary cytokine level measurement can be assessed feline CKD which is alternative and non-invasive method (Habenicht et al., 2013). Recently, the one study noted that clinicalpathologic disorders such as azothemia, hyperphosphatemia and anemia correlated with renal fibrosis in cats with CKD (McLeland et al., 2015).

Feline CKD are staged using International Renal Interest Society (IRIS; www.iris-kidney.com) classifications according to plasma creatinine concentration that can be

divided into 4 stages (<1.6 mg/dl, stage1; 1.6–2.8 mg/dl, stage 2; 2.9–5.0 mg/dl, stage 3; and >5.0 mg/dl, stage 4) (Elliott and Watson, 2009). In addition, IRIS classified feline CKD cases are sub-staged base on proteinuria and systemic blood pressure. Clinical progression, or stage is associated with increased mortality (McLeland et al., 2015). Prognosis for cats with CKD is good to poor depending on the IRIS CKD stage of the patient. (Polzin, 2013). The classification of CKD according to IRIS guideline is an important approach to managing CKD in cats (Elliott and Watson, 2009). Cats with CKD cannot be cured, the aim of treatment can only improve quality of life. The ideal treatment for feline CKD would be to correct the underlying cause of CKD and limit the progressive loss of renal function (Korman and White, 2013).

The most common kidney pathological lesion of feline CKD is chronic tubulointerstitial nephritis and fibrosis (DiBartola et al., 1987; Grauer, 2009; McLeland et al., 2015) and frequently reported pathological diagnosis is renal tubulointerstitial fibrosis (Lawson et al., 2015). The most cases of CKD cats the underlying etiology is unknown (Reynolds and Lefebvre, 2013). The pathogenesis of feline CKD include chronic kidney injury that can induce irreversible inflammation (Reynolds and Lefebvre, 2013). Many cytokines secreted by cells *such as* transforming growth factor-β1 (TGF-β1) that initiates renal inflammation and fibrogenesis (Arata et al., 2005; Bobkova et al., 2006). Studies were reported that urinary TGF-β1 levels in CKD cats were higher than healthy cats (Arata et al., 2005; Habenicht et al., 2013). Chronic hypoxia is likely to be another important in the pathogenesis of feline CKD which is associated with oxidative stress (Keegan and Webb, 2010). Studies had been indicated that cats with CKD had significantly higher oxidative stress than the clinically normal cats (Keegan and Webb, 2010; Piyarungsri et al., 2014). The renin-angiotensin-aldosterone system (RAAS) has been implicated in the pathogenesis of kidney disease. The study in cats with experimentally induced CKD showed that RAAS was upregulated and increased plasma renin activity, angiotensin I, angiotensin II, and aldosterone (Watanabe and Mishina, 2007). Moreover, hyperfiltration, proteinuria, tubulointerstitial inflammation, oxidative damage and induction of the RAAS are major factors conduce to the process of tubulointerstitial injury (Habenicht et al., 2013).

The major etiology of feline CKD remains unknown. Renal fibrosis is the final common outcome of all chronic progressive kidney disease (Huang et al., 2014c). At present, there are no effective treatments to slow the progression of renal fibrosis in cats. Therefore, researcher has focus on identifying risk factors which drives the progression of renal fibrosis for identify potential therapeutic targets (Lawson et al., 2015).

2.2. Role of Doxorubicin (DOX) in CKD

Doxurubicin (DOX) or anthracycline antibiotic is a chemotherapy drug, with cytotoxic and anti-proliferative properties (Lown, 1993). The toxicity of DOX is the effect on renal tissue. In renal tubular cell, doxorubicin-induced cell apoptosis has been reported in both in vivo and in vitro studies. Degeneration of tubular epithelial renal cells and a severe increase in serum creatinine was found in patients who receiving doxorubicin (Burke et al., 1977). Study in rats found that DOX induced toxicity in kidneys is associated with apoptosis of renal tubular cell (Zhang et al., 1996). Experimental CKD models were induced by the intravenous injection of DOX in rats (Yoneko et al., 2007). In vitro, DOX induced apoptosis in human renal cell line was also demonstrated (Chaotham et al., 2013). Renal tubular cell apoptosis plays a role in the pathophysiology of renal disease in human (Hauser and Oberbauer, 2002). The induction of renal tubular cell apoptosis provides a mechanism for the pathogenesis of renal tubular atrophy, which is the characteristic of CKD (Khan et al., 1999; Schelling and Cleveland, 1999). The main factor of doxorubicin-induced nephrotoxicity was the oxidative stress. DOX can activate O_2 molecule to create reactive oxygen species (ROS) including superoxide anion, hydrogen peroxide and hydroxyl radicals (Muller et al., 1997). One study indicated that the increased of oxidative stress and eNOS gene and protein expression were found in DOX-induced feline kidney cell line (Piyarungsri et al., 2014).

2.3. Potassium channel KCa3.1

Many types of ion channels are related to the kidney disease. Potassium channel is one of the channel reported to be involved with kidney disease (Bertuccio

and Devor, 2015). Recently, there are several studies investigate whether intermediate-conductance Ca^{2+}-activated K^+ channel KCa3.1 (KCa3.1) play a role in renal fibrosis (Grgic et al., 2009a; Mene and Pirozzi, 2010; Huang et al., 2013; Mene and Pirozzi, 2013; Huang et al., 2014d; Huang et al., 2015). KCa3.1 channel is called IKCa1, IK1, SK4 and KCNN4 (Wulff and Castle, 2010; Chen et al., 2016).

The structure of KCa3.1 subunit is composed of six hydrophobic alpha helical transmembrane spanning domains (S1-S6) and between S5-S6 located the pore loop for potassium ion efflux (Balut et al., 2012; Mene and Pirozzi, 2013; Morales et al., 2013). Four of these structures are assembled tetramerically to form an ion channel with the central pore (Neylon et al., 1999). The pore region contains potassium ion selective amino acid sequence GYG for highly selective for K^+ (Neylon et al., 1999) (Figure 1). This channel is Ca^{2+} activated channel which opens in response to an increase in cytoplasmic Ca^{2+} concentration (Balut et al., 2012). Activated KCa3.1 can cause membrane hyperpolarization and an increase Ca^{2+} entry into cells which creates an increase in the intracellular Ca^{2+} concentration (Mene and Pirozzi, 2010). Moreover, KCa3.1 channel is associated with the protein calmodulin (CaM), which is Ca^{2+} sensitivity of KCa3.1 gating process (Mene and Pirozzi, 2010; Morales et al., 2013). This channel regulates potassium outflow, the membrane potential and calcium-dependent cell functions including activation, migration, apoptosis and proliferation (Bradding and Wulff, 2009; Wulff and Castle, 2010; Shao et al., 2011). This channel is present in various cells in renal fibrotic process, including proximal tubular cells, fibroblasts, T-lymphocytes, macrophages and endothelial cells (Huang et al., 2014c). KCa3.1 channel is a therapeutic target for a variety of disease in human such as hematology disease such as sickle cell anemia (Balut et al., 2012), inflammatory diseases such as inflammatory bowel disease (Lam and Wulff, 2011), rheumatoid arthritis (Kang et al., 2014), respiratory disease such as asthma (Bradding and Wulff, 2009), cystic fibrosis and chronic obstructive pulmonary disease (Balut et al., 2012), allergic rhinitis (Lin et al., 2014), cardiovascular disease such as atherosclerosis and restenosis (Lam and Wulff, 2011), neulological disorders such as sclerosis (Lam and Wulff, 2011), cancer (Freise et al., 2013), renal fibrosis (Mene and Pirozzi, 2010; Huang et al., 2015).

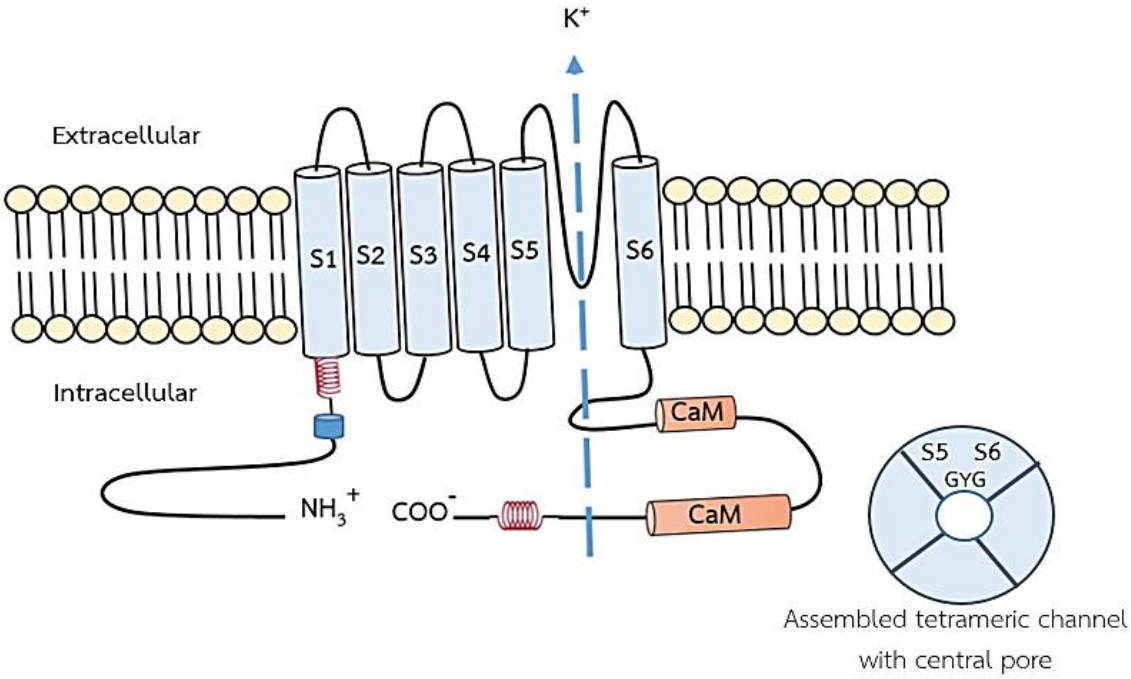


Figure 1 The structural model of KCa3.1 channel
(Adapted from Neylon et al.,1999; Mene and Pirozzi, 2013; Morales et al., 2013)

KCa3.1 channel was associated with kidney disorders including polycystic kidney disease (Albaqumi et al., 2008), renal fibrosis (Mene and Pirozzi, 2010), diabetic nephropathy (Huang et al., 2017), kidney allograft rejection (Grgic et al., 2009b), progressive kidney disease (Huang et al., 2015), acute kidney injury (Chen et al., 2016) and CKD (Bertuccio and Devor, 2015). Grgic et al. (2009) noted that increasing of KCa3.1 expression was found in unilateral ureteral obstruction (UUO) using mice as a model of renal fibrosis and CKD, and found that KCa3.1 play a role in renal fibroblast proliferation and fibrogenesis (Grgic et al., 2009a). KCa3.1 mediates TGF-β1 induce fibrosis and inflammation in human proximal tubular cells (Huang et al., 2013; Huang et al., 2014a). Recent study reported that KCa3.1 might be involved in renal tubular cell apoptosis (Chen et al., 2016). Moreover, KCa3.1 play a role endothelium-dependent vasodilation in kidney associated with hypertension which cause the development of CKD (Simonet et al., 2012). Blockade of KCa3.1 can reduce the fibrotic and inflammatory processes in the kidney of rat and mice model and human kidney cells (Bertuccio and Devor, 2015; Huang et al., 2015). In addition, Kang et al. (2014) stated that KCa3.1 inhibitor reduces experimental glomerulonephritis in rat

nephrotoxic nephritis model. Therefore, KCa3.1 is relate to pathophysiogical role kidney disease. Recent study suggested that KCa3.1 channel is a new target of CKD treatment in human patients and KCa3.1 inhibitors such as TRAM-34 may be a potential future renal therapeutics for CKD (Bertuccio and Devor, 2015).

2.4. Triarylmethane-34 (TRAM-34)

Triarylmethane-34 or TRAM-34 (1-[(2-Chlorophenyl) (diphenyl) methyl]-1*H*-pyrazole) is a selective KCa3.1 channel blocker that can inhibit KCa3.1 (Olivan-Viguera et al., 2013) TRAM-34 is a synthesized triarylmethane that is the close analogues of antimycotic clotrimazole and free of cytochrome P450 inhibition (Wulff et al., 2000; Jensen et al., 2002; Wulff and Castle, 2010). Nevertheless, study reported that TRAM-34 can inhibit several cytochrome P450 in rat and human (Agarwal et al., 2013). TRAM-34 has a high affinity blocker of KCa3.1 to both in normal physiology and disease (Wulff et al., 2000; Wulff et al., 2007; Balut et al., 2012). Moreover, TRAM-34 improves the metabolic stability and more potent (IC_{50}=20nM) than clotrimazole (IC_{50}=25-387 nM) (Triggle, 1999; Wulff et al., 2000). TRAM-34 is an inner pore blockers which it interacts with Val275 in S6 and Thr250 in the pore loop of KCa3.1 channel (Wulff and Castle, 2010). Olivan-Viguera et al. (2013) reported that TRAM-34 can block KCa current in mouse fibroblast cell line by patch-clamp recordings. Previous study suggested that TRAM-34 can attenuate renal fibrosis in murine renal fibroblast cell line and UUO-induced mice (Grgic et al., 2009a). In diabetic nephropathy mice, TRAM-34 can suppress the development of renal fibrosis and reduce fibrotic marker such as collagen type I, III and IV, fibronectin, α-smooth muscle actin, vimentin and fibroblast-specific protein-1 and reduce inflammatory cytokine such as TGF-β1, MCP-1 and chemokine ligand 20 (CCL20) (Huang et al., 2013; Huang et al., 2014c; Huang et al., 2014b; Huang et al., 2014d). In human proximal tubular cells, TRAM-34 can reduce TGF-β1 induced MCP-1 expression through Smad3, p38 and ERK1/2 signaling pathways (Huang et al., 2014a). Bertuccio and Devor (2015) suggested that TRAM-34 may has a protective effect for diabetic nephropathy through inhibition of NF-kB signaling pathway. Moreover, TRAM-34 can protect ciaplatin-induced renal tubular cell apoptosis in human proximal tubular epithelial cells and kidneys of mice model (Chen et al., 2016). TRAM-

34 was reported to be safe and well-tolerated in vitro and mice model (Bertuccio and Devor, 2015). The chemical structure of DOX was shown (Figure 2).

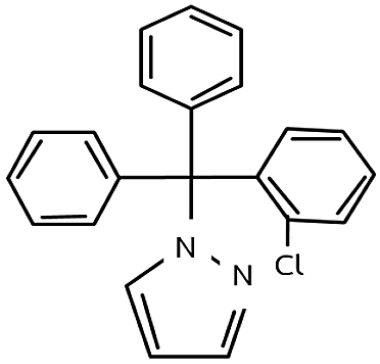


Figure 2 The chemical structure of TRAM-34
(Adapted from Jensen et al., 2002; Wulff and Castle, 2010)

However, the role of KCa3.1 channels in cats with CKD is unknown. There are no studies to investigate the effect of KCa3.1 potassium channels in cats with CKD. Investigation of the effect of KCa3.1 potassium channels in doxorubicin-induced feline kidney cells may lead to a better understanding of feline CKD pathophysiology through experimental study. Better knowledge about the pathophysiology of feline CKD and the role of KCa3.1 channel in this disease may lead us to a new therapeutic approach and better prognosis for feline CKD in the future.

CHAPTER III

MATERIALS AND METHODS

3.1. Conceptual framework of this study

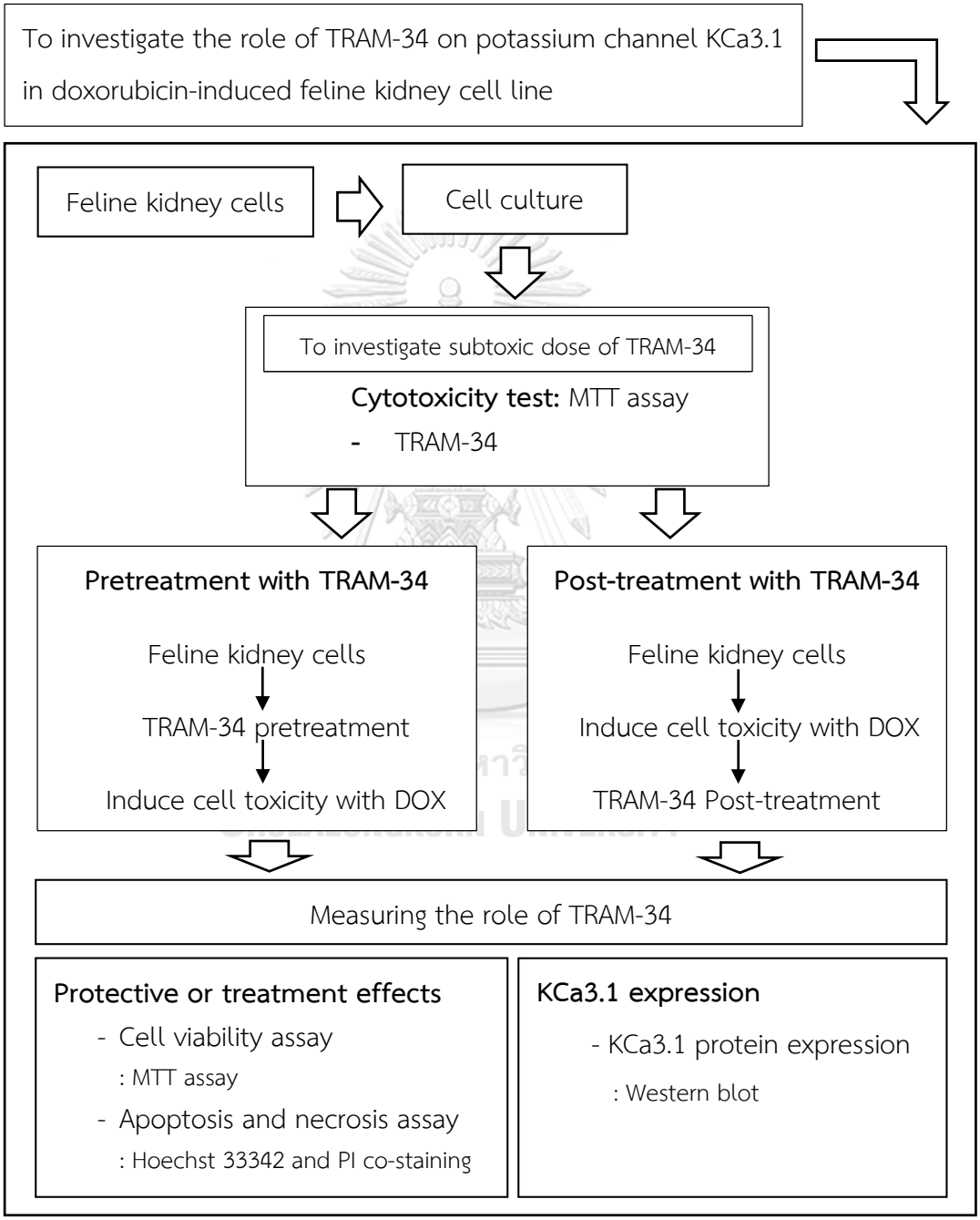


Figure 3 The conceptual framework of this study

3.2. Cell culture

The feline kidney cells (CRFK ATCC® CCL-94™) were used. CRFK cells are Crandell-Reese feline kidney cells. These feline kidney cells are epithelial originated from cortex part of kidney from 12 weeks old normal female domestic cats (*Felis catus*) (Crandell et al., 1973). The culture properties of these cells are adherent type. The cells grow as a monolayer of epithelial-like cells. The culture method was followed manufacturer's protocol. Cells in TC-dish was cultured in Dulbecco's Modified Eagle Medium (DMEM) and supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 µg/ml streptomycin, L-alanyl-L-glutamine and non-essential amino acids solution. Cells was incubated under a humidified atmosphere consisting of 5% CO_2 and 95% air at 37 °C in incubator.

The cells were subcultured and changed the medium every 2 days as follows: the culture medium was removed and the cell monolayers was washed with phosphate buffered saline (PBS) and trypsinized with 0.25% (w/v) Trypsin-0.53 mM EDTA solution and observed cells under an inverted microscope until cell layer was dispersed. The cells were transferred to centrifuge tube and centrifuged at approximately 125 x g for 5 minute (min) to form a cell pellet. The supernatant in centrifuge tube was removed. Complete growth medium (DMEM+10% FBS) about 7 to 9 ml was added and homogenized. Cell suspension was dispensed to new culture vessels. These cells in TC-dish was incubated for 2 days at 37 °C in humidified atmosphere consisting of 5% CO_2 in incubator. All experiments were performed at passage 190-203.

3.3. Study designs

3.3.1. Cytotoxicity test of TRAM-34 in feline kidney cells

Subtoxic dose of TRAM-34 in feline kidney cells was investigated by cytotoxicity test of TRAM-34 (Figure 3). TRAM-34 (1-[(2-Chlorophenyl) (diphenyl) methyl]-1H-pyrazole) is a potent selective KCa3.1 channel blocker prepared in dimethyl sulfide (DMSO) as a vehicle. Subtoxic concentration of TRAM-34 in feline kidney cells was first clarified for investigating the effect of TRAM-34 on DOX-induced cell toxicity. TRAM-34 was obtained from Sigma, Catalog No.T6700. TRAM-34 was dissolved by DMSO to

provide the stock solution of TRAM-34 (at 10 mM) (Olivan-Viguera et al., 2013; Chen et al., 2016). DMSO was obtained from VWR, Catalog No. 67685. Feline kidneys cells were added with different concentrations of TRAM-34 (0.1, 0.5, 1, 5, 10, 25, 50 and 100 µM) and incubated at different time periods (24, 48 and 96 hour (h)). The negative control cells had only culture medium without any treatments. The DMSO control cells were added with DMSO (at same amount added dose of TRAM-34). The viability of treated, negative control and DMSO control were measured. Cells were serum-free starved 16 h before adding TRAM-34 or DMSO.

Cells were verified using 3-(4,5-Dimethylthaiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT) (Sigma, St Louis, USA) viability assay as follows: Feline kidney cells in TC-dish were trypsinized with 0.25% (w/v) Trypsin-0.53 mM EDTA solution and then the cells were transferred to centrifuge tube and centrifuged at approximately 125 x g for 5 min to form a cell pellet. The supernatant in centrifuge tube was removed. Cell pellet of feline kidney cells were diluted to 1 ml with culture medium. Cells suspension (1×10^4 cells/well) was transferred to 48-well plate and incubated at 37 °C for 2-3 days to 70% confluent. Cells were treated with different concentrations of TRAM-34 or DMSO (0, 0.1, 0.5, 1, 5, 10, 25, 50 and 100 µM) and incubated at different time periods (24, 48 and 96 h). Cell medium in a 48-well plate were removed and incubated with MTT (5 mg/ml) for 3 h at 37 °C. The MTT solution was removed and then added 100 µl of DMSO to each well to dissolve formazan crystals. The samples in a 48-well plate were transfered to a 96-well plate. The OD of samples in a 96-well plate were determined by using spectrophotometry at 570 nm (ELx800, Biotek, Vermont, USA) and recorded the absorbance results.

The highest TRAM-34 concentration with no differences in cell viability between the control cells and cells treated with TRAM-34 was considered as the subtoxic dose. This part of the study was independent experimental in triplicate and repeated at three times.

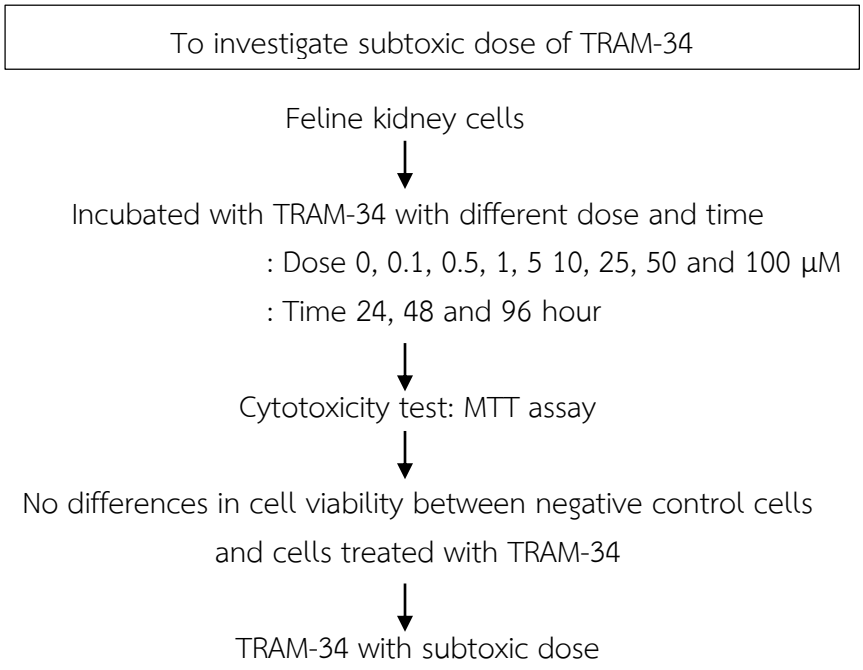


Figure 4 Cytotoxicity test of TRAM-34 in feline kidney cells

3.3.2. Pretreatment with TRAM-34

Feline kidney cells were divided into four groups according to the experimental designs of pretreatment with TRAM-34 (Figure 4). All groups of feline kidney cell line were cultured with the same media and environment. The cells were seeded at an optimal density in multi-well plates and incubated for 24 h. Cells were starved in serum-free medium 16 h for growth-arrested cells before used in experimental (Huang et al., 2014a; Huang et al., 2014d; Chen et al., 2016).

1) Negative control group:

A normal feline kidney cells without any treatments which have only the culture medium served as the negative control.

2) Doxorubicin-treated control group:

DOX with appropriate concentration was added into the medium to induce renal injury (Chaotham et al., 2013). DOX was obtained from Merck Millipore, Catalog No.324380. DOX was dissolved by sterile water to provide the stock solution of DOX (8 mM). DOX with appropriate concentration was

added into feline kidney cell line and served as a DOX-treated control group. According to Piyarungsri and colleague (2014), DOX at 8 µM concentration for 48 h was selected as an appropriate dose and time to be used which caused 50% cell viability in cytotoxicity test of DOX in feline kidney cells. Therefore, the feline kidney cells of DOX-treated control group was added DOX at 8 µM for 48 h.

3) TRAM-34 pretreatment group:

The feline kidney cells were pretreated with TRAM-34 before DOX were added. According to Chen and colleague (2016), TRAM-34 pretreated for 30 min in HK-2 cells can protect cisplatin-induced renal cell injury (Chen et al., 2016). Feline kidney cells were firstly incubated with TRAM-34 at subtoxic dose for 30 min or 24 h and induced nephrotoxicity with 8 µM of DOX for 48 h.

4) DMSO pretreatment control group:

DMSO control for pretreatment group was added with only DMSO (at same amount added subtoxic dose of TRAM-34) 30 min or 24 h before 8 µM of DOX was added for 48 h.

All groups were measured for the cell viability, apoptosis and necrosis status and KCa3.1 protein expression. The cell viability assay was measured by using MTT assay. The mode of cell death analyzed by using 2'-(4'-ethoxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5'-bi-1H-benzimidazole trihydrochloridetrihydate(Hoechst 33342) (Sigma) and 3,8-Diamino-5-[3-(diethylmethylammonio)propyl]-6-phenylphenanthridinium diiodide (propidium iodide; PI) (Sigma) co-staining for apoptosis and necrosis assay. The expression of KCa3.1 channel was studied. Cells of each group was collected to test for the protein expression of KCa3.1 channel and measured by using western blot assay (Figure 4). This study was independent experimental in triplicate and repeated at least three times. The results from all groups were compared with the statistical method.

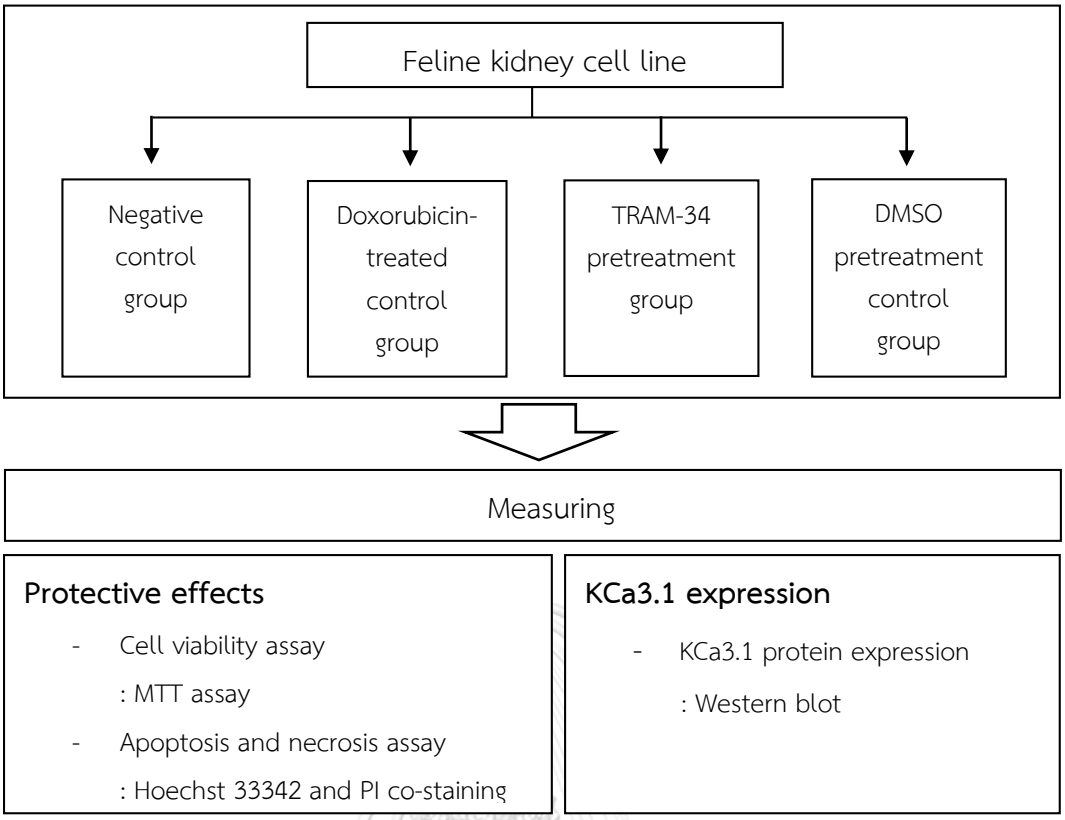


Figure 5 Experimental designs of TRAM-34 pretreatment and control groups of DOX-induced cell toxicity in feline kidney cells

3.3.3. Post-treatment with TRAM-34

Feline kidney cells were randomly divided to four groups (Figure 5). All groups of feline kidney cell line was cultured with the same media and environment. The cells were seeded at an optimal density in multi-well plates and incubated for 24 h. The cells were starved in serum-free medium 16 h before use in experiment (Huang et al., 2014a; Huang et al., 2014d; Chen et al., 2016).

1) Negative control group:

A normal feline kidney cells without any treatments was served as the negative control.

2) Doxorubicin-treated control group:

The cells of DOX-treated control group were added with doxorubicin at 8 μM for 48 h to induced cell injury.

3) TRAM-34 post-treatment group:

Feline kidney cells were induced nephrotoxicity with 8 µM of DOX for 48 h and then incubated with TRAM-34 at subtoxic dose for 24 h.

4) DMSO post-treatment control group:

DMSO control for treatment group was added with DMSO only (at same amount added subtoxic dose of TRAM-34) for 24 h after 8 µM of DOX was added for 48 h.

All groups were measured the cell viability, apoptosis and necrosis status and KCa3.1 protein expression. The cell viability assay measured by using MTT assay. The mode of cell death analyzed by using Hoechst 33342 and PI co-staining for apoptosis and necrosis assay. KCa3.1 protein expression measured by using western blot (Figure 6). This part of the study was independent experimental in triplicate and repeated at least three times.

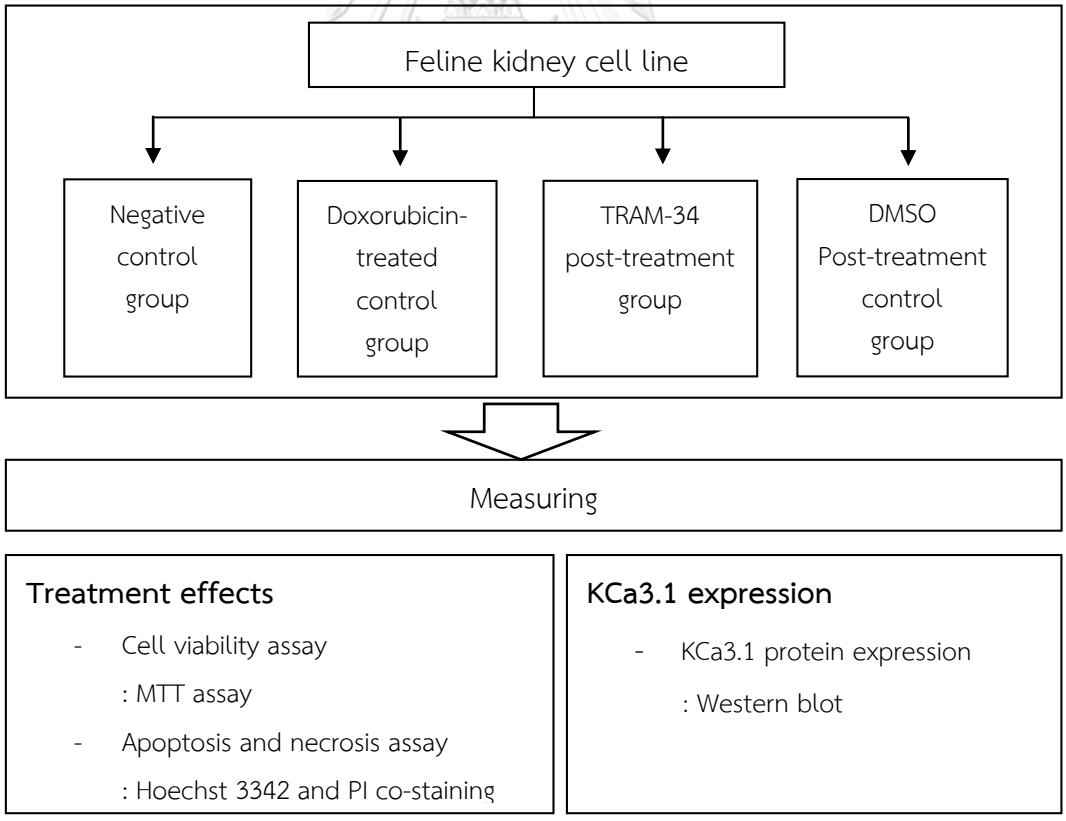


Figure 6 Experimental designs of TRAM-34 post-treatment and control groups of DOX-induced cell toxicity in feline kidney cells

3.4. Laboratory examination

3.4.1. MTT assay

The kidney cells were collected to screen for cell viability by MTT assay. The modification of Mosmann's method used for the MTT colorimetric assay (Mosmann, 1983). The principle of MTT assay, the yellow colored MTT was reduced by mitochondrial succinate dehydrogenase to purple colored formazan crystals.

MTT assay measured for cell viability as follows: feline kidney cells (1×10^4 cells/well) were cultured in a 48-well plate at 37 °C, and treated with different concentrations of TRAM-34 or DMSO and/or DOX for different times of incubation follow each experimental group. Negative control group is the feline kidney cells without any treatments. Cell culture media in a 48-well plate were removed and cells were incubated with MTT (5 mg/ml) for 3 h at 37 °C. MTT solution was removed and added 100 µl of DMSO to each well to dissolve formazan crystals. The samples in 48-well plate was transferred to 96-well plate. The samples in a 96-well plate were determined by using spectrophotometry at 570 nm (ELx800, Biotek, Vermont, USA) and recorded absorbance results.

The relative cell viability (%) was presented as a percentage relative to the negative control group.

3.4.2. Apoptosis and necrosis assay

Detection and measurement of cell death by fluorescence microscopy of apoptosis and necrosis use Hoechst 33342 and PI co-staining. Apoptosis cells have cell shrinkage, membrane blebbing, fragmented nuclei, condensed chromatin and fragmentation of chromosomal DNA. Hoechst 33342 is a blue-fluorescence dye specific for bind to intracellular DNA, can be used to identify apoptotic cells and normal cell. Biochemical actions of Hoechst 33342 is membrane-permeable and fluorescent DNA stains that intercalate in A-T regions of DNA. Hoechst 33342 can penetrate through normal and apoptotic cell membranes, but Hoechst 33342 stains the condensed chromatin in apoptotic cells more brightly than the chromatin in normal cells. Thus, the apoptotic cells with condensed chromatin and/or fragmented nuclei is stained

with Hoechst 33342. Hoechst 33342 is soluble in sterile water at concentration of 20 mg/ml.

When necrosis occurs, the cells have loss the integrity of the cell membranes. PI is phenanthridinium intercalator fluorescent stain for nucleic acids. PI is a red-fluorescence dye and stains necrotic cells by binding to DNA in the nucleus. PI dye cannot penetrate through normal cell membranes, but can enter through necrotic cell membranes because it has membrane impermeant property. Cell membrane integrity excludes PI from staining viable and apoptotic cells. PI is dissolved in PBS at concentration of 1 mg/ml.

All cells including normal, apoptosis and necrotic cells can stain with Hoechst 33342. PI can only stain necrosis cells or dead cells. Apoptosis and normal cells can be identified by the morphology of cells and apoptotic cell has blue fluorescence intensity by Hoechst 33342 higher than normal cells.

Hoechst 33342 and PI co-staining were used for measurement of mode of cell death as follows: feline kidney cells were cultured in a 24-well plate and pretreated with TRAM-34 for 30 min or 24 h or post-treatment with TRAM-34 for 24 h on DOX-induced cell toxicity for 48 h. Negative control group was the feline kidney cells without any treatments. The cells were fixed with 4% formaldehyde for 20 min at room temperature, blocked with 1% bovine serum albumin (BSA) for 1 h at room temperature. Then cells were washed with PBS 5 min 3 times. Cells were stained with 2 µg/ml of the Hoechst 33342 for 15 min in dark room temperature and then washed with PBS 5 min 3 times. Cells were stained with 2 µg/ml of the PI dry for 20 min in dark room temperature. Then the cells were washed with PBS 5 min 3 times and mounted with 1:10 glycerol.

Each well was visualized in 5 fields under a fluorescence microscope (Mbq 52 ac with Axiovert 40, Zeiss). Then calculation of the mean of the percentage of apoptosis cells and necrosis cell were reported as percentages of apoptotic cells respect to the total and percentages of PI positive cells respect to the total, respectively. Percentages of apoptotic cells respect to the total and percentages of PI positive cells respect to the total were determined according to equation 1 and 2

Percentages of apoptotic cells respect to the total (%)

$$= \frac{\text{Apoptotic cells}}{\text{All cells}} \times 100 \quad \text{(equation 1)}$$

Percentages of PI positive cells respect to the total (%)

$$= \frac{\text{Necrotic cells}}{\text{All cells}} \times 100 \quad \text{(equation 2)}$$

Normal cells were live cells with normal nuclei that have blue chromatin with organized structure. Apoptosis cells were live cells with apoptotic nuclei with bright blue chromatin that is highly condensed or fragmented. Necrotic cells were cells with red chromatin.

3.4.3. KCa3.1 protein expression

In this study, western blot assay was used for detection of KCa3.1 protein expression as follows: feline kidney cells were lysed with cytobuster protein extraction reagent (Novagen®) and were prepared through centrifugation at 16,000 x g for 5 min at 4 °C. The supernatant of cell lysates were quantified using the Bradford protein assay (Bio-Rad®). Protein extract 30 grams/ml was added with 5% B-mercaptoethanol in loading buffer. The samples were heated with dry heat-plate for 5 min at 95 °C and then spun down for 5 min. 12% separating gel was prepared by sterile water 3.3 ml, 30% acrylamide mix (29.2% acrylamide and 0.8% N,N'-methylene-bis-acrylamide) 4 ml, 1.5 M tris (pH 8.8) 2.5 ml, 10% sodium dodecyl sulfate (SDS) 0.1 ml, 10% ammonium persulfate (APS) 0.1 ml and tetramethylethylenediamine (TEMED) 0.006 ml. The separating gel was added into the glass plates from the casting frame. The stacking gel which includes sterile water 2.2 ml, 30% acrylamide mix 0.67 ml, 0.5 M tris (pH 6.8) 1 ml, 10% SDS 0.04 ml, 10% APS 0.04 ml and TEMED 0.006 ml, was added on top of the solid separating gel. The marker and protein samples loaded into wells. Proteins were separated using SDS-Polyacrylamide gel electrophoresis (PAGE) and transferred onto polyvinylidene fluoride (PVDF) membrane (Life Technologies) by using TE70x semi-dry transfer unit (Hoefer). SNAP i.d.® 2.0 protein detection system (EMD

Milipore) was used for western blotting of this experiment. Each membrane was washed 3 times with tris-buffered saline with Tween 20 (TBS-T buffer). The blots were blocked with SNAP i.d.® immunoblot blocking reagent (EMD Milipore) for 10 min at room temperature. Membrane incubated with primary antibodies against KCa3.1 (primary rabbit polyclonal anti-KCNN4) obtained from Abcam, Catalog No. ab215990 at 1:3000 dilution and β-actin (primary rabbit polyclonal anti-β-actin) (ab199799, Abcam) at 1:1000 dilution (Table1) for 18 h at 4 °C. Each membrane was washed 3 times with TBS-T buffer. Then the membranes were incubated with horseradish peroxidase-conjugated secondary goat anti-rabit antibody (HAF008, R&D system Inc.) at 1:2000 dilution for 10 min at room temperature (Table1). Each membrane was washed 3 times with TBS-T buffer. Then membrane was inserted in the cassette. Chemiluminescent HRP detection reagent (EMD Milipore) added on the membrane. Then X-ray film touched with each membrane in the cassette. X-ray films developed by using manual processing (developing, fixing and washing process) in dark room. Picture films scanned to computer and then ImageJ 1.51j8 software were used to quantify the expression of KCa3.1 based on the intensity of the bands. The ratio of KCa3.1/β-actin was shown the results of quantifying densitometry of KCa3.1 protein expression.

Table 1 Primary and secondary antibodies of KCa3.1 (as KCNN4)

Protein name	Antibodies	Dilution
KCNN4	primary rabbit polyclonal anti-KCNN4	1:3000
	secondary goat anti-rabbit	1:2000
β-actin	primary rabbit polyclonal anti-β-actin	1:1000
	secondary goat anti-rabbit	1:2000

3.5. Statistical analysis

Results were reported as mean ± SEM. Statistical analyses were performed using the SPSS software (version 16). Normality of the distribution of data and homogeneity of variances was assessed by using Shapiro-Wilk test and Levene test, respectively. Statistical analysis of data from multiple groups were analyzed by one-way analysis of variance (ANOVA), followed by Bonferroni post-hoc test if equal

variances or Games-Howell post-hoc test if unequal variances. Statistical analysis of data from two groups were analyzed by independent Student's t-test. The statistical significant level of this study was considered as $p<0.05$.

CHAPTER IV
RESULTS

4.1. Cytotoxicity test of TRAM-34 in feline kidney cells

To investigate that subtoxic doses of TRAM-34 treatment does not affect feline kidney cell lines. The cells were treated with various concentrations of TRAM-34 (0, 0.1, 0.5, 1, 5, 10, 25, 50 and 100 μM) at different time periods (24, 48 and 96 h). The cytotoxicity results showed no significantly differences in cell viability between the negative control and cells treated with TRAM-34 from 0.1 to 100 μM for 24 h after treatment ($p>0.05$). Therefore, TRAM-34 from the concentration of 0.1 to 100 μM were not toxic to feline kidney cells for 24 h after treatment (Table 2; Figure 7). The DMSO-control at 100 μM for 24 h had significantly lower percentage of cell viability than the negative control and the concentration of 0.1, 1 and 5 μM ($p<0.05$) (Table 3; Figure 8). The concentration of TRAM-34 at 100 μM was the subtoxic dose of TRAM-34 for 24 h in feline kidney cells when compared with the negative control.

For 48 h treated with TRAM-34, the percent of viability results showed no significantly differences between the negative control and cells treated with TRAM-34 from 0.1 to 50 μM after treatment. Percent viabilities at 100 μM of TRAM-34 was significantly lower than percentage of cell viability of the negative control, 0.1 and 1 μM concentrations of TRAM-34 ($p<0.05$) (Table 2; Figure 7). The DMSO-control at 100 μM for 48 h had significantly lower percent of viable cells than at the concentration of 0, 1 and 5 μM ($p<0.05$) (Table 3; Figure 8). Therefore, a significant reduction of cell surviving of feline kidney cells treated with TRAM-34 was seen in this study started at 100 μM for 48 h. The percent viability of feline kidney cells decreased when increased concentration of TRAM-34 and DMSO (Table 3; Figure 8). Concentration-dependent reduction in cell viability was detected at 48 h after treatment with TRAM-34 and DMSO. Thus, feline kidney cells treated with TRAM-34 from 0.1 to 50 μM concentrations were not toxic to feline kidney cells at 48 h. We found the subtoxic dose of TRAM-34 in feline kidney cells as the concentration 50 μM for 48 h when compared with the negative control.

For 96 h treated with TRAM-34, the cells treated with TRAM-34 at 0.1 to 25 μM concentration were not significantly differences in cell viability when compared with the negative control. Cells treated with TRAM-34 at 50 and 100 μM for 96 h had significantly lower percentage of cell viability than the negative control and the concentration of TRAM-34 at 0.1, 1, 5 and 10 μM (p<0.05). Moreover, percent viabilities at 25 μM of TRAM-34 for 96 h was significantly lower than percent viability at 0.1, 1 and 5 μM of TRAM-34 (p<0.05) (Table 2; Figure 7). The DMSO-control at 100 μM for 96 h had significantly lower percent viability than at the concentration of 0, 0.1, 1, 5 and 10 μM (p<0.05). In addition, the DMSO-control at 25 μM for 96 h had significantly lower percent of cell viability than at the concentration of 0.1 μM and the negative control (p<0.05). The DMSO-control at 100 μM for 96 h had significantly lower percent of viable cells than at the concentration of 0, 0.1, 1, 5 and 10 μM (p<0.05) (Table 3; Figure 8). The percent viability of feline kidney cells at 96 h after treatment also decreased when increased concentration of TRAM-34 and DMSO (Table 3; Figure 8). Therefore, feline kidney cells treated with TRAM-34 from 0.1 to 25 μM concentrations were not toxic to feline kidney cells for 96 h. The concentration of TRAM-34 at 25 μM was the subtoxic dose of TRAM-34 for 96 h in feline kidney cells when compared with the negative control.

Table 2 Percent (%) viability by MTT method of feline kidney cells at various concentrations of TRAM-34 (0 – 100 μM) treatment for 24, 48 and 96 h

TRAM-34 (μM)	Time (h)		
	24 (n=3)	48 (n=3)	96 (n=3)
Negative control	100	100	100
0.1	109.363 ± 2.057	102.010 ± 2.368	102.698 ± 2.698
0.5	102.730 ± 4.673	92.939 ± 3.942	98.262 ± 0.760
1	102.501 ± 3.355	99.108 ± 0.946	101.900 ± 3.973
5	97.445 ± 2.430	100.736 ± 6.763	102.114 ± 1.604
10	101.466 ± 0.790	93.069 ± 2.642	97.471 ± 1.377
25	95.208 ± 3.567	82.706 ± 3.174	86.499 ± 3.876^{abc}
50	89.517 ± 5.629	82.778 ± 3.622	81.907 ± 3.299*^{abcd}
100	90.637 ± 4.199	76.844 ± 1.486*^{ab}	76.056 ± 2.088*^{abcd}

h = hour

*p < 0.05 when compared with negative control

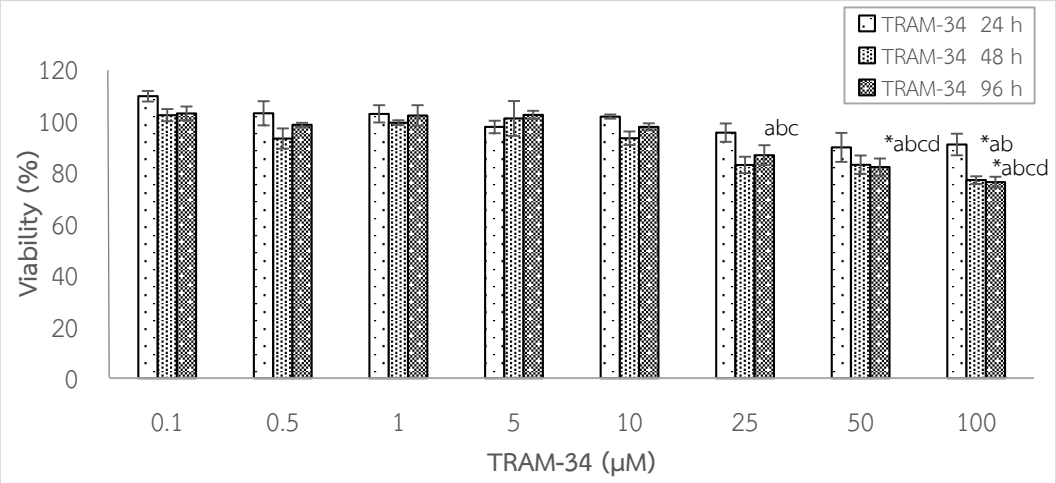
^{a}p < 0.05 when compared with concentration at 0.1 μM

^{b}p < 0.05 when compared with concentration at 1 μM

^{c}p < 0.05 when compared with concentration at 5 μM

^{d}p < 0.05 when compared with concentration at 10 μM

Figure 7 Cytotoxicity of TRAM-34 by MTT method of TRAM-34 treatment at 0 to 100 μM concentrations for 24, 48 and 96 h in feline kidney cells



h = hour

*p < 0.05 when compared with negative control

[a]p < 0.05 when compared with concentration at 0.1 μM

[b]p < 0.05 when compared with concentration at 1 μM

[c]p < 0.05 when compared with concentration at 5 μM

[d]p < 0.05 when compared with concentration at 10 μM

Table 3 Percent (%) viability by MTT method of feline kidney cells at 0 to 100 μM concentrations of TRAM-34 or DMSO-control for 24, 48 and 96 h

TRAM-34 Dose (μM)	Time (h)					
	TRAM-34 24 h (n=3)	DMSO-control 24 h (n=3)	TRAM-34 48 h (n=3)	DMSO-control 48 h (n=3)	TRAM-34 96 h (n=3)	DMSO-control 96 h (n=3)
Negative control	100	100	100	100	100	100
0.1	109.363 ± 2.057	99.587 ± 3.829	102.010 ± 2.368	95.298 ± 3.256	102.698 ± 2.698	100.185 ± 0.473
0.5	102.730 ± 4.673	97.459 ± 2.262	92.939 ± 3.942	92.997 ± 2.971	98.262 ± 0.760	97.376 ± 1.850
1	102.501 ± 3.355	100.379 ± 1.441	99.108 ± 0.946	98.500 ± 0.137	101.900 ± 3.973	96.092 ± 2.758
5	97.445 ± 2.430	94.219 ± 1.054	100.736 ± 6.763	89.369 ± 1.566	102.1137 ± 1.604	93.011 ± 3.336
10	101.466 ± 0.790	89.376 ± 1.082	93.069 ± 2.642	81.697 ± 1.972	97.471 ± 1.377	87.440 ± 1.599
25	95.208 ± 3.567	85.359 ± 3.908	82.706 ± 3.174	82.184 ± 5.917	86.499 ± 3.876^{abc}	79.308 ± 0.983^{*a}
50	89.517 ± 5.629	85.381 ± 2.243	82.778 ± 3.622	79.312 ± 8.219	81.907 ± 3.299^{*abcd}	80.140 ± 3.569
100	90.637 ± 4.199	80.731 ± 0.829^{*abc}	76.844 ± 1.486^{*ab}	72.905 ± 1.431^{*bc}	76.056 ± 2.088^{*abcd}	43.720 ± 1.002^{*abcd}

h = hour

*$p < 0.05$ when compared with negative control

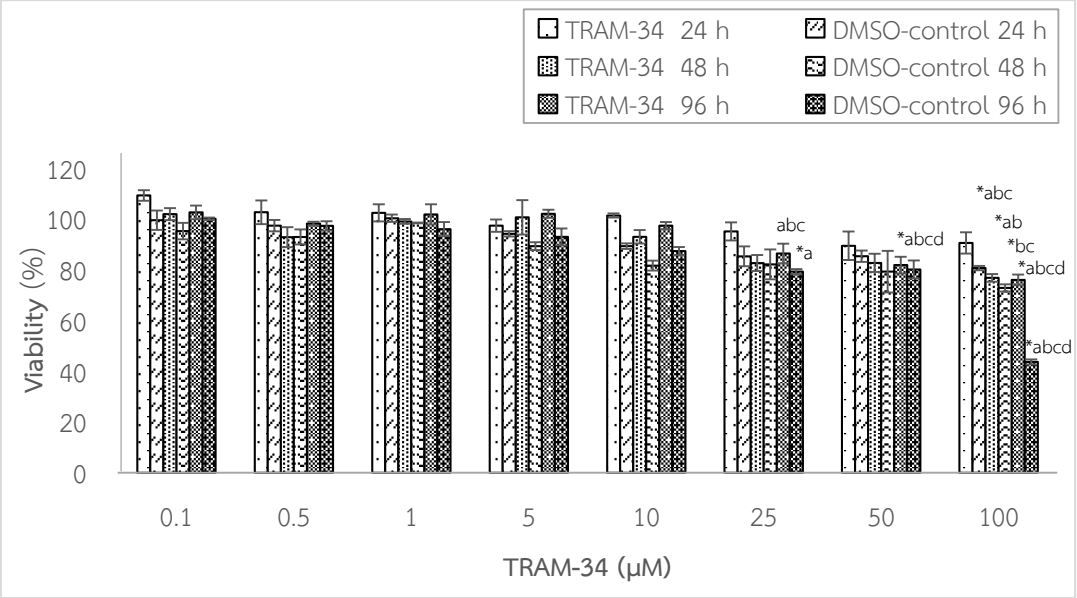
$^{a}p < 0.05$ when compared with concentration at 0.1 μM

$^{b}p < 0.05$ when compared with concentration at 1 μM

$^{c}p < 0.05$ when compared with concentration at 5 μM

$^{d}p < 0.05$ when compared with concentration at 10 μM

Figure 8 Percent (%) viability by MTT method of feline kidney cells at 0 to 100 μM concentrations of TRAM-34 or DMSO-control for 24, 48 and 96 h



h = hour

*$p < 0.05$ when compared with negative control

[a]$p < 0.05$ when compared with concentration at 0.1 μM

[b]$p < 0.05$ when compared with concentration at 1 μM

[c]$p < 0.05$ when compared with concentration at 5 μM

[d]$p < 0.05$ when compared with concentration at 10 μM

For compared between TRAM-34 and DMSO for each dose, The cytotoxicity results showed no significantly differences in cell viability between DMSO-control and cells treated with TRAM-34 at 0.1, 0.5, 1, 5, 25, 50 and 100 μM concentrations for 24 and 48 h (Table 4 and 5) and at 0.1, 0.5, 1, 5, 25 and 50 μM concentrations for 96 h after treatment ($p>0.05$) (Table 6). Percent viabilities at 10 μM of TRAM-34 for 24, 48 and 96 h was significantly higher than percent viability of DMSO-control ($p<0.05$) (Table 4, 5 and 6). In addition, The DMSO-control at 100 μM for 96 h had significantly lower percent viability than cells treated with TRAM-34 ($p<0.001$) (Table 6).

Table 4 Percent (%) viability by MTT method of feline kidney cells at 0 to 100 μM concentrations of TRAM-34 treatment for 24 h, compared with DMSO control

Dose (μM)	24 h		t-test
	TRAM-34 (n=3)	DMSO-control (n=3)	Significant (2-tailed)
0.1	109.363 ± 2.057	99.587 ± 3.829	0.088
0.5	102.730 ± 4.673	97.459 ± 2.262	0.367
1	102.501 ± 3.355	100.379 ± 1.441	0.592
5	97.445 ± 2.430	94.219 ± 1.054	0.290
10	101.466 ± 0.790	89.376 ± 1.082	0.001**
25	95.208 ± 3.567	85.359 ± 3.908	0.136
50	89.517 ± 5.629	85.381 ± 2.243	0.532
100	90.637 ± 4.199	80.731 ± 0.829	0.082

h = hour

**Student's t-test is significant at p<0.01 level (2-tailed)

Table 5 Percent (%) viability by MTT method of feline kidney cells at 0 to 100 μM concentrations of TRAM-34 treatment for 48 h, compared with DMSO control

Dose (μM)	48 h		t-test
	TRAM-34 (n=3)	DMSO-control (n=3)	Significant (2-tailed)
0.1	102.010 ± 2.368	95.298 ± 3.256	0.171
0.5	92.939 ± 3.942	92.997 ± 2.971	0.991
1	99.108 ± 0.946	98.500 ± 0.137	0.559
5	100.736 ± 6.763	89.369 ± 1.566	0.231
10	93.069 ± 2.642	81.697 ± 1.972	0.026*
25	82.706 ± 3.174	82.184 ± 5.917	0.942
50	82.778 ± 3.622	79.312 ± 8.219	0.719
100	76.844 ± 1.486	72.905 ± 1.431	0.129

h = hour

*Student's t-test is significant at p<0.05 level (2-tailed)

Table 6 Percent (%) viability by MTT method of feline kidney cells at 0 to 100 μM concentrations of TRAM-34 treatment for 96 h, compared with DMSO control

Dose (μM)	96 h		t-test
	TRAM-34 (n=3)	DMSO-control (n=3)	Significant (2-tailed)
0.1	102.698 ± 2.698	100.185 ± 0.473	0.451
0.5	98.262 ± 0.760	97.376 ± 1.850	0.681
1	101.900 ± 3.973	96.092 ± 2.758	0.296
5	102.1137 ± 1.604	93.011 ± 3.336	0.070
10	97.471 ± 1.377	87.440 ± 1.599	0.009**
25	86.499 ± 3.876	79.308 ± 0.983	0.147
50	81.907 ± 3.299	80.140 ± 3.569	0.735
100	76.056 ± 2.088	43.720 ± 1.002	0.000***

h = hour

**Student's t-test is significant at p<0.01 level (2-tailed)

***Student's t-test is significant at p<0.001 level (2-tailed)

4.2. Feline kidney cells pretreatment with TRAM-34 before DOX treatment

Feline kidneys cell were initially treated with subtoxic dose of TRAM-34 (0 – 25 μM) for 30 min and 24 h. Then, kidney cell toxicity was induced with 8 μM of DOX for 48 h. The protective effects and KCa3.1 protein expression were determined in this experiment.

4.2.1. Protective effects

Subtoxic doses of TRAM-34 were used to treat feline kidney cells before inducing nephrotoxicity with 8 μM of DOX for 48 h. The protective effects, which included cytotoxicity assay, apoptosis and necrosis assay were measured.

4.2.1.1. Cytotoxicity assay

Feline kidney cells were pretreated with various concentrations of TRAM-34 (0, 0.1, 0.5, 1, 5, 10 and 25 μM) for 30 min and 24 h before the induction of cell toxicity using 8 μM of DOX for 48 h.

For pretreatment 24 h, the results of pretreatment with TRAM-34 at 0.1, 0.5 and 1 μM concentrations showed significantly protective effect when compared with DOX-treated control ($p<0.05$). In addition, percent cell viability of DOX-treated control was significantly difference with the negative control in pretreatment 24 h ($p<0.05$) (Table 7; Figure 9).

For pretreatment 30 min, the results demonstrated no significant protective effect of pretreatment with TRAM-34 at 0.1 to 25 μM concentration when compared with DOX-treated control ($p>0.05$). In addition, pretreatment with TRAM-34 for 30 min at 5, 10 and 25 μM concentration had significantly lower percentage of cell viability than the negative control ($p<0.05$) (Table 7; Figure 9).

Table 7 Percent cell viabilities of pre-treatment with TRAM-34 for 30 min and 24 h in DOX (8 μM) induced feline kidney cell toxicity for 48 h

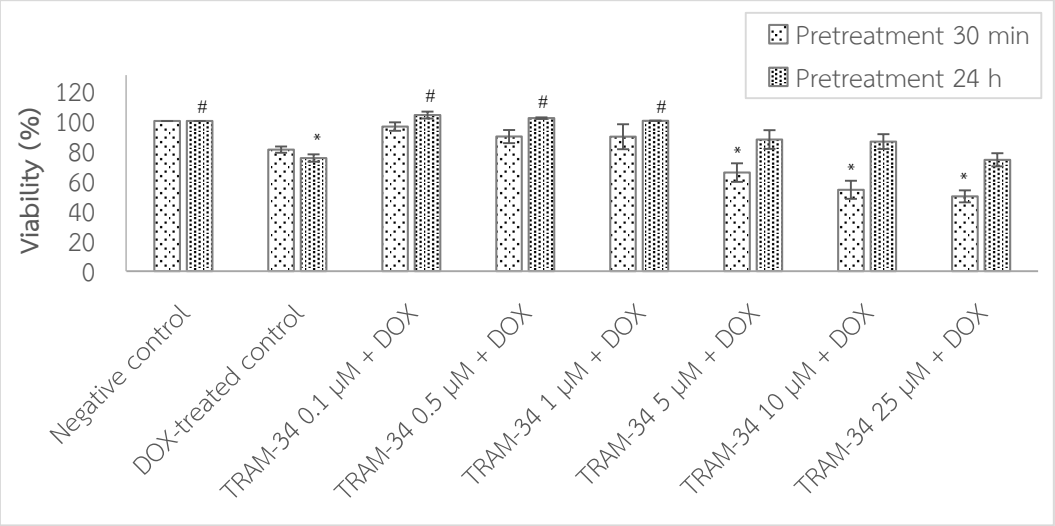
TRAM-34 (μM)	% cell viability	
	Pretreatment 30 min (n=3)	Pretreatment 24 h (n=3)
Negative control	100	100[#]
DOX-treated control	80.971 ± 2.10	75.327 ± 2.438*
0.1	96.335 ± 2.826	103.970 ± 2.288[#]
0.5	89.646 ± 4.332	101.938 ± 0.542[#]
1	89.556 ± 8.310	100.097 ± 0.154[#]
5	65.660 ± 6.149*	87.642 ± 6.236
10	54.160 ± 6.041*	86.291 ± 4.967
25	49.853 ± 3.996*	74.159 ± 4.349

DOX = doxorubicin, h = hour

*$p < 0.05$ when compared with negative control

[#]$p < 0.05$ when compared with DOX-treated control

Figure 9 Percent cell viabilities of pre-treatment with TRAM-34 for 30 min and 24 h in DOX (8 μM) induced feline kidney cell toxicity for 48 h



DOX = doxorubicin, h = hour

*$p < 0.05$ when compared with negative control

#$p < 0.05$ when compared with DOX-treated control

For DMSO-control, pretreatment 24 h with DMSO-treated control from 0.1 to 25 μM concentrations and the pretreatment 30 min with 0.1 concentration had significantly lower percentage of cell viability than the negative control (p<0.05) (Table 8; Figure 10). In addition, both pretreatment 24 h and 30 min demonstrated that the DOX-treated control cell was not significantly differences in cell viability with DMSO-control from 0.1 to 25 μM concentrations (p>0.05) (Table 8; Figure 10). Therefore, the results indicated no significant protective effect of pretreatment 24 h or 30 min with DMSO-treated control when compared with DOX-treated control.

Table 8 Percent cell viabilities of the pretreatment with TRAM-34 or DMSO-control for 30 min and 24 h in DOX (8 μM) induced feline kidney cell toxicity for 48 h

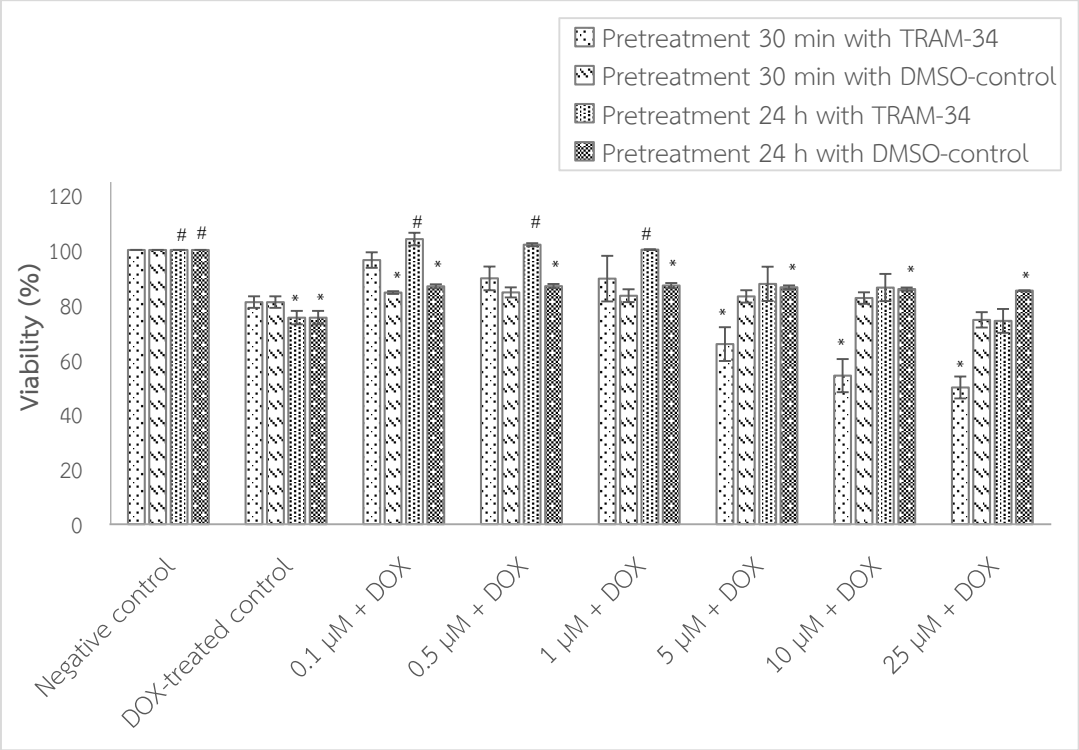
Groups	% cell viability			
	Pretreatment 30 min		Pretreatment 24 h	
	TRAM-34 (n=3)	DMSO-control (n=3)	TRAM-34 (n=3)	DMSO-control (n=3)
Negative control	100		100#	
DOX-treated control	80.971 ± 2.10		75.327 ± 2.438*	
0.1 μM	96.335 ± 2.826	84.465 ± 0.483*	103.970 ± 2.288#	86.745 ± 0.717*
0.5 μM	89.646 ± 4.332	84.524 ± 1.819	101.938 ± 0.542#	86.816 ± 0.759*
1 μM	89.556 ± 8.310	83.322 ± 2.345	100.097 ± 0.154#	87.015 ± 0.925*
5 μM	65.660 ± 6.149*	83.079 ± 2.254	87.642 ± 6.236	86.411 ± 0.670*
10 μM	54.160 ± 6.041*	82.466 ± 2.095	86.291 ± 4.967	85.731 ± 0.607*
25 μM	49.853 ± 3.996*	74.504 ± 2.814	74.159 ± 4.349	85.153 ± 0.083*

DOX = doxorubicin, h = hour

*p < 0.05 when compared with negative control

#p < 0.05 when compared with DOX-treated control

Figure 10 Percent cell viabilities of pretreatment with TRAM-34 or DMSO-control for 30 min and 24 h in DOX (8 μM) induced feline kidney cell toxicity for 48 h



DOX = doxorubicin, h = hour

*$p < 0.05$ when compared with negative control

#$p < 0.05$ when compared with DOX-treated control

For compared between TRAM-34 and DMSO for each dose, percent viabilities of pretreatment with TRAM-34 at 0.1, 0.5 and 1 μM concentrations for 24 h was significantly higher than percent viability of DMSO-control (p<0.01) (Table 9). In addition, The cytotoxicity results showed no significantly differences in cell viability between DMSO-control and cells pretreated with TRAM-34 at 5, 10 and 25 μM concentrations for 24 h (p>0.05) (Table 9). Pretreatment 30 min with TRAM-34 at 0.1 μM had significantly increased percent cell viability when compared with DMSO-control (p<0.05) (Table 10). Moreover, percent viability of cells pretreated with TRAM-34 at 10 and 25 μM concentrations for 30 min was significantly decreased when compared with DMSO-control (p<0.01) (Table 10).

Table 9 Percent cell viabilities of pretreatment with TRAM-34 for 24 h in DOX (8 μM) induced feline kidney cell toxicity for 48 h, compared with DMSO control

Dose (μM)	Pretreatment 24 h		t-test
	TRAM-34 (n=3)	DMSO-control (n=3)	Significant (2-tailed)
0.1	103.970 ± 2.288	86.745 ± 0.717	0.002**
0.5	101.938 ± 0.542	86.816 ± 0.759	0.000***
1	100.097 ± 0.154	87.015 ± 0.925	0.000***
5	87.642 ± 6.236	86.411 ± 0.670	0.862
10	86.291 ± 4.967	85.731 ± 0.607	0.916
25	74.159 ± 4.349	85.153 ± 0.083	0.065

h = hour

**Student's t-test is significant at p<0.01 level (2-tailed)

***Student's t-test is significant at P<0.001 level (2-tailed)

Table 10 Percent cell viabilities of pretreatment with TRAM-34 for 30 min in DOX (8 μM) induced feline kidney cell toxicity for 48 h, compared with DMSO control

Dose (μM)	Pretreatment 30 min		t-test
	TRAM-34 (n=3)	DMSO-control (n=3)	Significant (2-tailed)
0.1	96.335 ± 2.826	84.465 ± 0.483	0.014*
0.5	89.646 ± 4.332	84.524 ± 1.819	0.337
1	89.556 ± 8.310	83.322 ± 2.345	0.509
5	65.660 ± 6.149	83.079 ± 2.254	0.056
10	54.160 ± 6.041	82.466 ± 2.095	0.011*
25	49.853 ± 3.996	74.504 ± 2.814	0.007**

h = hour

*Student's t-test is significant at $p<0.05$ level (2-tailed)

**Student's t-test is significant at $p<0.01$ level (2-tailed)

4.2.1.2. Apoptosis and necrosis assay

Hoescht 33342 and PI staining were used to determine cell death. The results of apoptosis and necrosis assay of the pretreatment with TRAM-34 at 0 to 25 μM concentrations for 30 min or 24 h before the induction of cell toxicity using 8 μM of DOX for 48 h were listed (Table 11; Figure 11). Apoptosis and necrosis cells, which have condensed and fragmented chromosome, were detected by staining with Hoechst 33342 and PI co-staining, respectively. For apoptosis, percentages of chromosome condensed cells with Hoechst 33342 staining were measured to determine apoptotic cells. The results of cell death from apoptosis were also reported by percentages of apoptotic cells respect to the total. For necrosis, percentages of chromosome condensed cells with PI co-staining were measured to determine necrotic cells. The results of cell death from necrosis were also shown by percentages of PI positive cells respect to the total.

Table 11 Percentages of apoptotic cells respect to the total and PI positive cells respect to the total of pretreatment with TRAM-34 at 0 to 25 μM concentrations for 30 min and 24 h before DOX-induced feline kidney cells toxicity for 48 h

TRAM-34 pretreatment groups (μM)	Pretreatment 30 min		Pretreatment 24 h	
	% of apoptotic cells respect to the total (n=3)	% of PI positive cells respect to the total (n=3)	% of apoptotic cells respect to the total (n=3)	% of PI positive cells respect to the total (n=3)
Negative control	11.351 ± 0.419$^{\#}$	5.543 ± 0.066	5.652 ± 0.213$^{\#}$	2.297 ± 0.026
DOX-treated control	33.343 ± 0.216**	18.447 ± 0.977	25.863 ± 0.788*	6.539 ± 0.328
0.1	35.289 ± 0.744**	18.049 ± 0.924	15.263 ± 0.790$^{\#}$	5.949 ± 0.398
0.5	38.210 ± 1.897	20.917 ± 0.848*	15.168 ± 0.565*$^{\#}$	5.895 ± 0.581
1	37.005 ± 2.031	19.913 ± 0.479*	15.131 ± 0.557*$^{\#}$	6.028 ± 0.319
5	39.346 ± 1.253*	31.604 ± 2.838	27.690 ± 0.501**	7.003 ± 0.371
10	46.756 ± 0.864**$^{\#}$	41.725 ± 1.831*$^{\#}$	31.473 ± 0.931*	10.037 ± 0.367*
25	51.270 ± 0.204**$^{\#\#}$	48.343 ± 0.570**$^{\#\#}$	40.780 ± 4.499	18.623 ± 1.304

DOX = Doxorubicin, h = hour

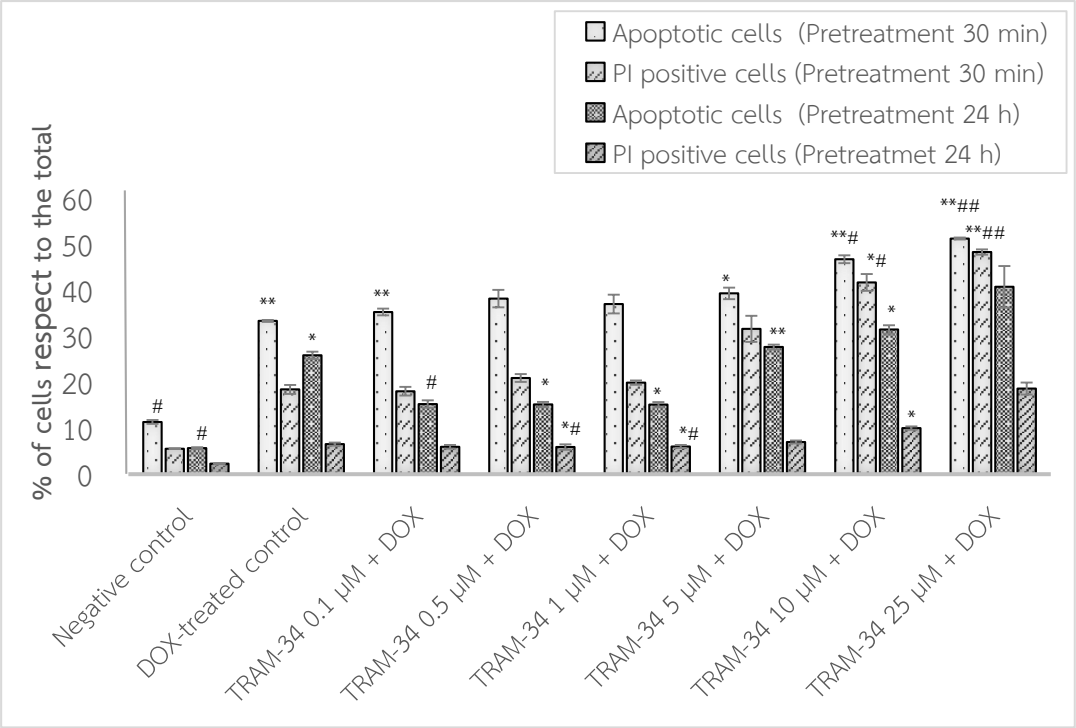
*$p < 0.05$ when compared with negative control

$^{\#}p < 0.05$ when compared with DOX-treated control

**$p < 0.01$ when compared with negative control

$^{\#\#}p < 0.01$ when compared with DOX-treated control

Figure 11 Percentages of apoptotic cells respect to the total and PI positive cells respect to the total of feline kidney cells were treated with TRAM-34 at 0 to 25 μM concentrations for 30 min or 24 h before induced cell toxicity with DOX for 48 h



DOX = Doxorubicin, h = hour

*$p < 0.05$ when compared with negative control

#$p < 0.05$ when compared with DOX-treated control

**$p < 0.01$ when compared with negative control

##$p < 0.01$ when compared with DOX-treated control

The results of pretreatment with TRAM-34 for 24 h at 0.5, 1, 5 and 10 μM concentrations and DOX-treated control had significantly higher percentage of apoptotic cells respect to the total than the negative control (p<0.05). In addition, percentages of apoptotic cells respect to the total for DOX-treated control was significantly difference with the negative control and TRAM at 0.1, 0.5 and 1 μM concentrations in pretreatment 24 h (p<0.05). Pretreatment 24 h with 0.1, 0.5 and 1 μM of TRAM-34 had significantly lower percentages of apoptotic cells respect to the total than DOX-treated control (p<0.05) (Table 12; Figure 12 and 13). Therefore, pretreatment with 0.1 to 1 μM of TRAM-34 for 24 h reduced cellular apoptosis in DOX-induced feline kidney cells. Moreover, pretreatment with TRAM-34 at 10 μM concentrations were significant higher in necrosis cells than negative control (Table 12; Figure 12 and 13).

Table 12 Percentages of apoptotic cells respect to the total and PI positive cells respect to the total of pretreatment with TRAM-34 at 0 to 50 μM concentrations for 24 h before DOX-induced feline kidney cells toxicity for 48 h

TRAM-34 pretreatment groups (μM)	Pretreatment 24 h	
	% of apoptotic cells respect to the total (n=3)	% of PI positive cells respect to the total (n=3)
Negative control	5.652 ± 0.213$^{\#}$	2.297 ± 0.026
DOX-treated control	25.863 ± 0.788*	6.539 ± 0.328
0.1	15.263 ± 0.790$^{\#}$	5.949 ± 0.398
0.5	15.168 ± 0.565*$^{\#}$	5.895 ± 0.581
1	15.131 ± 0.557*$^{\#}$	6.028 ± 0.319
5	27.690 ± 0.501**	7.003 ± 0.371
10	31.473 ± 0.931*	10.037 ± 0.367*
25	40.780 ± 4.499	18.623 ± 1.304

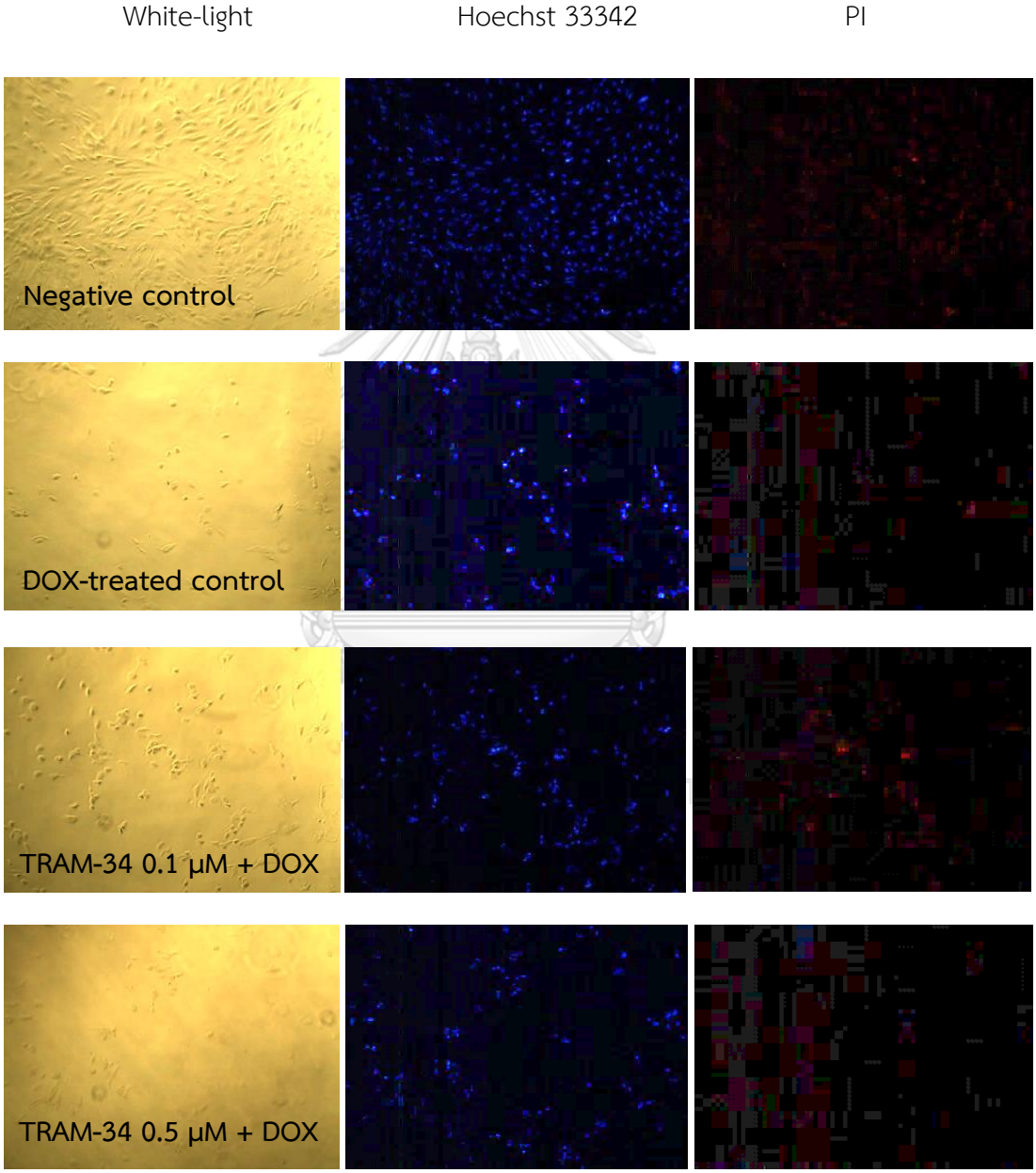
DOX = Doxorubicin, h = hour

*p < 0.05 when compared with negative control

$^{\#}p$ < 0.05 when compared with DOX-treated control

**p < 0.01 when compared with negative control

Figure 12 Feline kidney cells were treated with TRAM-34 at 0 to 25 μM concentrations for 24 h before induced cell toxicity with DOX for 48 h. Apoptotic cells displayed cells with nuclear condensation stained in bright blue fluorescence and necrotic cells demonstrated damaged cells stained in red fluorescence.



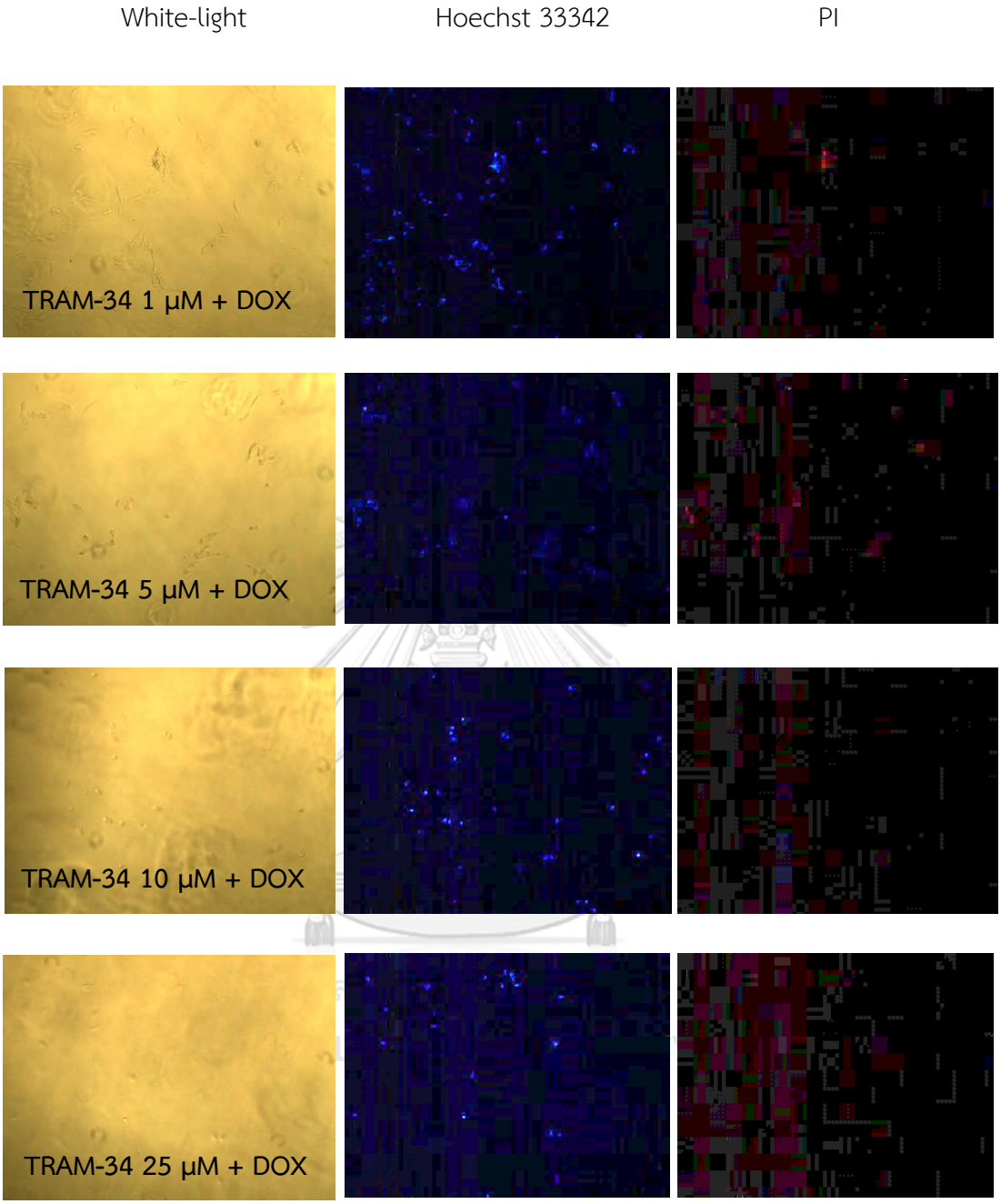
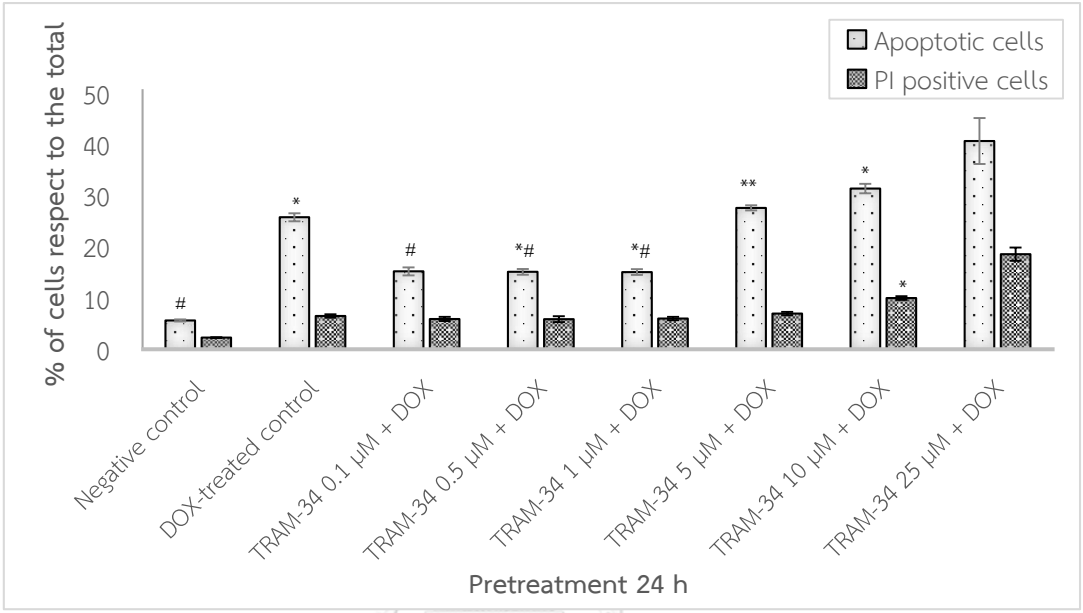

White-light
Hoechst 33342
PI
TRAM-34 1 μM + DOX
TRAM-34 5 μM + DOX
TRAM-34 10 μM + DOX
TRAM-34 25 μM + DOX

Figure 13 Percentages of apoptotic cells respect to the total and Percentages of PI positive cells respect to the total of feline kidney cells were treated with TRAM-34 at 0 to 25 μM concentrations for 24 h before induced cell toxicity with DOX for 48 h.



DOX = Doxorubicin

*$p < 0.05$ when compared with negative control

#$p < 0.05$ when compared with DOX-treated control

**$p < 0.01$ when compared with negative control

For pretreatment 30 min, the results demonstrated significant increase in percentage of apoptotic cells respect to the total of pretreatment with TRAM-34 at 0, 10 and 25 μM concentrations when compared with DOX-treated control (p<0.05). In addition, pretreatment with TRAM-34 at 0.1, 5, 10 and 25 μM concentration and DOX-treated control had significantly higher apoptosis cells than the negative control (Table 13; Figure 14 and 15). Likewise, the results of percentages of PI positive cells respect the total for pretreatment 30 min with TRAM-34 at 10 and 25 μM concentration was significant higher than DOX-treated control (p<0.05). In addition, pretreatment with TRAM-34 at 0.5, 1, 10 and 25 μM concentration had significantly higher in necrosis cells than the negative control (p<0.05) (Table 13; Figure 14 and 15).

Table 13 Percentages of apoptotic cells respect to the total and PI positive cells respect to the total of pretreatment with TRAM-34 at 0 to 25 μM concentrations for 30 min before DOX-induced feline kidney cells toxicity for 48 h

TRAM-34 pretreatment groups (μM)	Pretreatment 30 min	
	% of apoptotic cells respect to the total (n=3)	% of PI positive cells respect to the total (n=3)
Negative control	11.351 ± 0.419$^{\#}$	5.543 ± 0.066
DOX-treated control	33.343 ± 0.216^{a}	18.447 ± 0.977
0.1	35.289 ± 0.744^{a}	18.049 ± 0.924
0.5	38.210 ± 1.897	20.917 ± 0.848*
1	37.005 ± 2.031	19.913 ± 0.479*
5	39.346 ± 1.253*	31.604 ± 2.838
10	46.756 ± 0.864**$^{\#}$	41.725 ± 1.831*$^{\#}$
25	51.270 ± 0.204**$^{\#\#}$	48.343 ± 0.570**$^{\#\#}$

DOX = Doxorubicin, h = hour

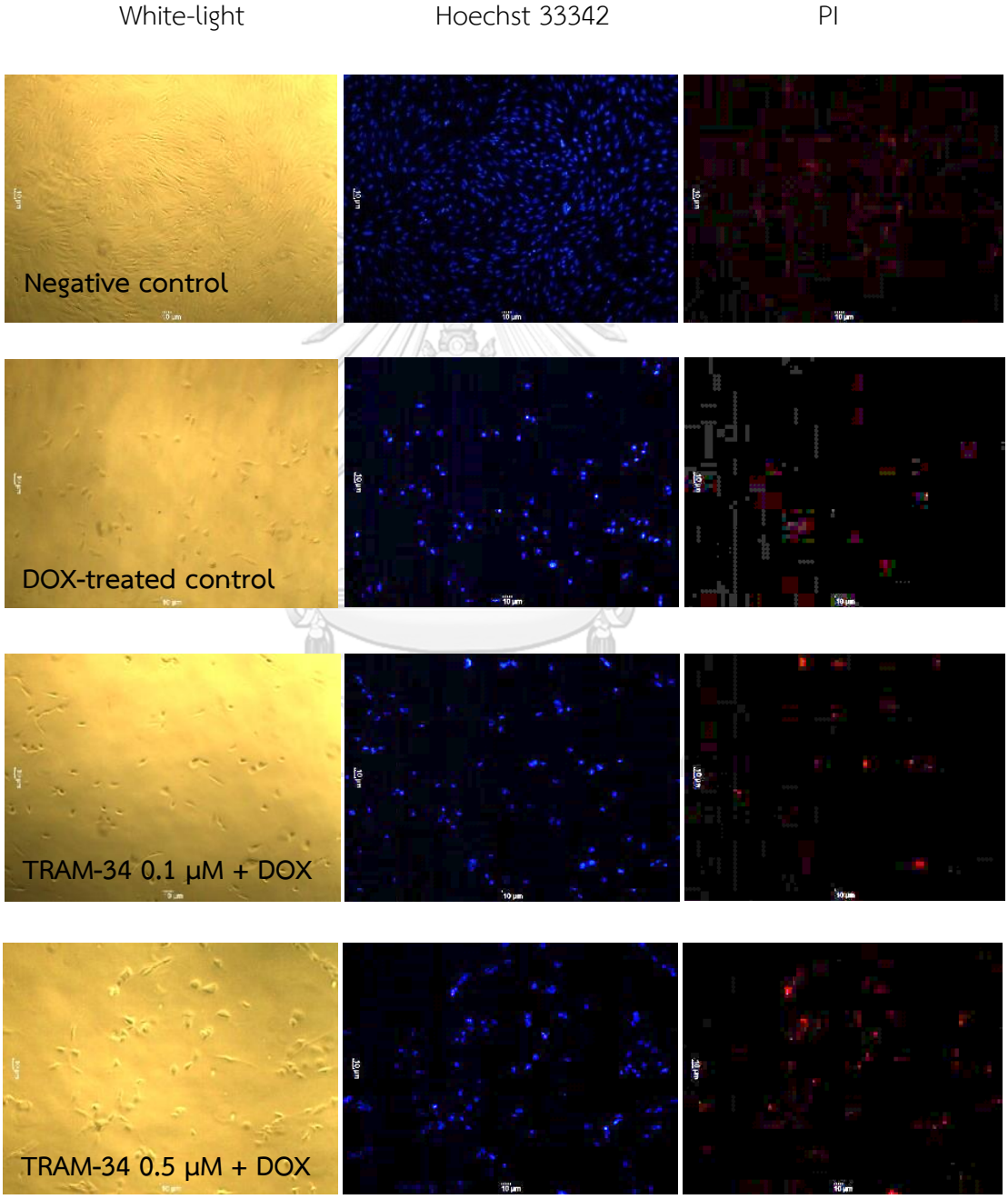
*p < 0.05 when compared with negative control

$^{\#}p$ < 0.05 when compared with DOX-treated control

**p < 0.01 when compared with negative control

$^{\#\#}p$ < 0.01 when compared with DOX-treated control

Figure 14 Feline kidney cells were treated with TRAM-34 at 0 to 25 µM concentrations for 30 min before induced cell toxicity with DOX for 48 h. Apoptotic cells displayed cells with nuclear condensation stained in bright blue fluorescence and necrotic cells demonstrated damaged cells stained in red fluorescence.



White-light Hoechst 33342 PI

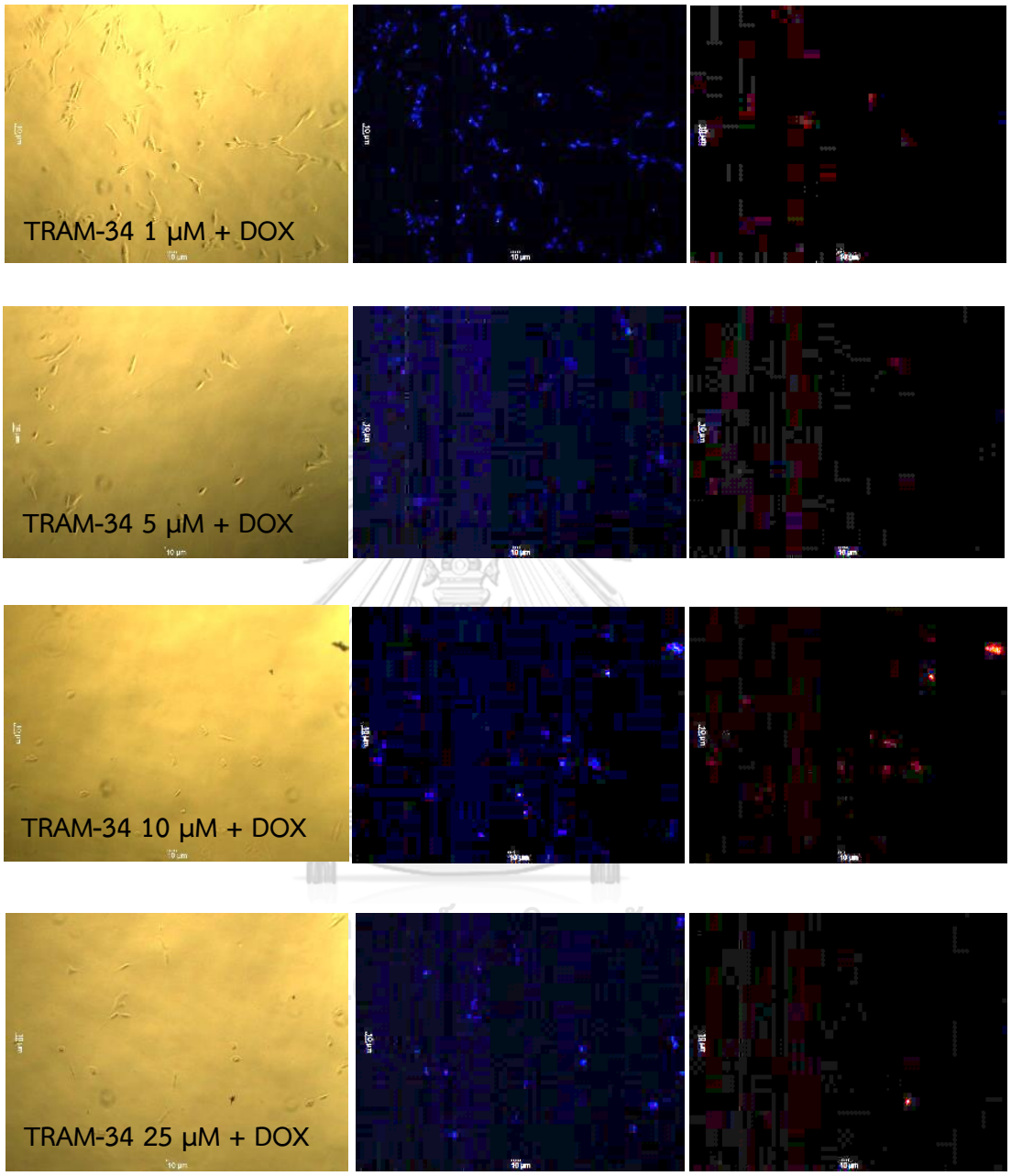
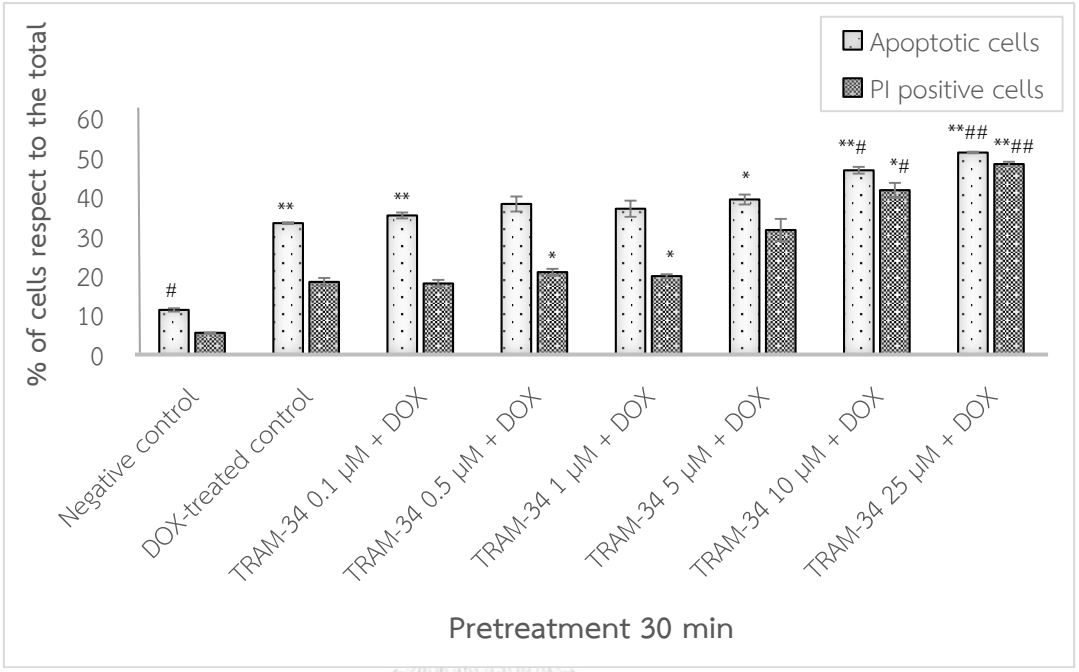


Figure 15 Percentages of apoptotic cells respect to the total and Percentages of PI positive cells respect to the total of feline kidney cells were treated with TRAM-34 at 0 to 25 μM concentrations for 30 min before induced cell toxicity with DOX for 48 h.



DOX = Doxorubicin

*$p < 0.05$ when compared with negative control

#$p < 0.05$ when compared with DOX-treated control

**$p < 0.01$ when compared with negative control

##$p < 0.01$ when compared with DOX-treated control

For DMSO control, the results of pretreatment for 24 h with DMSO-treated control at 0.5, 1, 5, 10 and 25 μM concentrations and pretreatment for 30 min with 0.1, 0.5. 1, 10 and 25 μM concentrations had significantly higher percentage of apoptotic cells respect to the total than the negative control ($p<0.05$). In addition, both pretreatment for 24 h and 30 min with DMSO-treated control found that DOX-treated control was significantly difference in percentage of apoptotic cells respect to the total with the negative control ($p<0.05$). Pretreatment for 24 h with DMSO-treated control at 10 to 25 μM concentrations had significantly increased cellular apoptosis when compared with DOX-treated control ($p<0.05$) (Table 14; Figure 14).

For necrosis, the results of pretreatment for 24 h and 30 min with DMSO-treated control 10 μM concentrations were significant higher percentage of PI positive cells respect to the total than the negative control ($p<0.05$). Likewise, pretreatment for 30 min with DMSO-treated control at 0.5, 1 and 25 μM concentrations was significantly difference in necrotic cells with the negative control ($p<0.05$). Pretreatment for 30 min with DMSO-treated control at 10 and 25 μM concentrations had significantly higher percentage of PI positive cells respect to the total than DOX-treated control ($p<0.05$) (Table 14; Figure 14).

Table 14 Percentages of apoptotic cells respect to the total and PI positive cells respect to the total of pretreatment with TRAM-34 or DMSO-control at 0 to 25 μM concentrations for 30 min or 24 h before DOX-induced feline kidney cells toxicity for 48 h

TRAM-34 pretreatment groups (μM)	Pretreatment 30 min				Pretreatment 24 h			
	% of apoptotic cells respect to the total		% of PI positive cells respect to the total		% of apoptotic cells respect to the total		% of PI positive cells respect to the total	
	TRAM-34 (n=3)	DMSO-control (n=3)	TRAM-34 (n=3)	DMSO-control (n=3)	TRAM-34 (n=3)	DMSO-control (n=3)	TRAM-34 (n=3)	DMSO-control (n=3)
Negative control	11.351 ± 0.419$^{\#}$	11.351 ± 0.419$^{\#}$	5.543 ± 0.066	5.543 ± 0.066	5.652 ± 0.213$^{\#}$	5.652 ± 0.213$^{\#}$	2.297 ± 0.026	2.297 ± 0.026
DOX-treated control	33.343 ± 0.216**	33.343 ± 0.216**	18.447 ± 0.977	18.447 ± 0.977	25.863 ± 0.788*	25.863 ± 0.788*	6.539 ± 0.328	6.539 ± 0.328
0.1	35.289 ± 0.744**	33.747 ± 0.804**	18.049 ± 0.924	18.542 ± 1.047	15.263 ± 0.790$^{\#}$	25.173 ± 1.256	5.949 ± 0.398	6.396 ± 0.292
0.5	38.210 ± 1.897	33.834 ± 0.804**	20.917 ± 0.848*	19.256 ± 0.118**	15.168 ± 0.565*$^{\#}$	25.262 ± 1.089*	5.895 ± 0.581	6.375 ± 0.365
1	37.005 ± 2.031	35.404 ± 1.036*	19.913 ± 0.479*	20.094 ± 0.558*	15.131 ± 0.557*$^{\#}$	26.650 ± 0.491**	6.028 ± 0.319	6.555 ± 1.503
5	39.346 ± 1.253*	40.669 ± 1.956	31.604 ± 2.838	31.676 ± 1.866	27.690 ± 0.501**	30.628 ± 1.355*	7.003 ± 0.371	7.905 ± 0.863
10	46.756 ± 0.864**$^{\#}$	49.724 ± 0.543**$^{\#\#}$	41.725 ± 1.831*$^{\#}$	40.058 ± 1.430*$^{\#}$	31.473 ± 0.931*	33.223 ± 0.823**	10.037 ± 0.367*	10.474 ± 0.472*
25	51.270 ± 0.204**$^{\#\#}$	52.604 ± 0.395**$^{\#\#}$	48.343 ± 0.570**$^{\#\#}$	51.561 ± 0.191**$^{\#}$	40.780 ± 4.499	38.561 ± 1.709*	18.623 ± 1.304	21.456 ± 1.620

DOX = Doxorubicin, h = hour

*$p < 0.05$ when compared with negative control

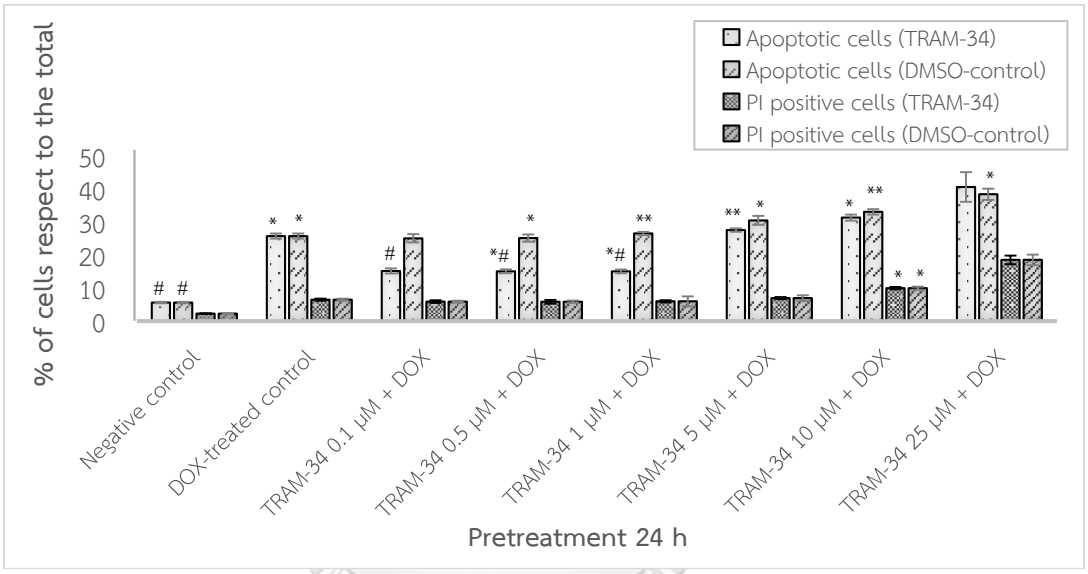
$^{\#}p < 0.05$ when compared with DOX-treated control

**$p < 0.01$ when compared with negative control

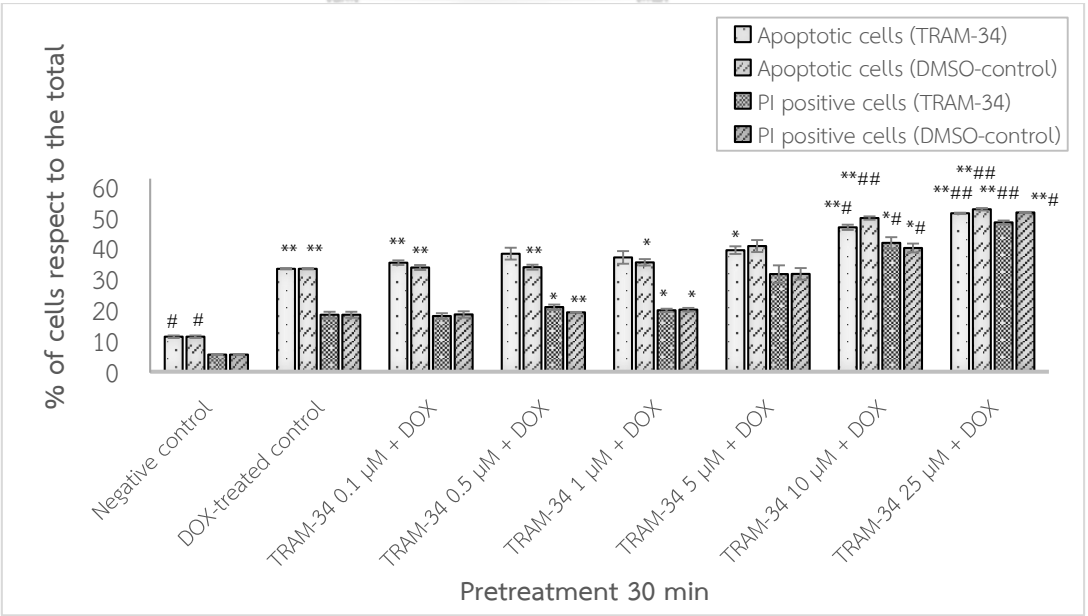
$^{\#\#}p < 0.01$ when compared with negative control

Figure 16 Percentages of apoptotic cells respect to the total and PI positive cells respect to the total of pretreatment with TRAM-34 or DMSO-control at 0 to 25 μM concentrations before DOX-induced feline kidney cells toxicity for 48 h. (A) Pretreatment for 24 h (B) Pretreatment for 30 min. *p < 0.05 when compared with negative control; #p < 0.05 when compared with DOX-treated control; **p < 0.01 when compared with negative control; ##p < 0.01 when compared with DOX-treated control.

A



B



For compared between TRAM-34 and DMSO for each dose, Percentages of apoptotic cells respect to the total of pretreatment with TRAM-34 at 0.1, 0.5 and 1 μM concentrations for 24 h was significantly lower than percentages of apoptotic cells respect to the total of DMSO-control (p<0.05), but not significantly differences in percentages of PI positive cells respect to the total (p>0.05) (Table 15). In addition, The results showed no significantly differences in both percentages of apoptotic cells respect to the total and percentages of PI positive cells respect to the total between DMSO-control and cells pretreated with TRAM-34 at 5, 10 and 25 μM concentrations for 24 h (Table 15) (p>0.05).

Pretreatment 30 min with TRAM-34 at 0.1 to 25 μM concentrations was not significantly differences in both percentages of apoptotic cells respect to the total and percentages of PI positive cells respect to the total when compared with DMSO-control (p>0.05), expect percentages of PI positive cells respect to the total of TRAM-34 at 25 μM concentration for 30 min was significantly lower than DMSO-control (p<0.05) (Table 16).

Table 15 Percentages of apoptotic cells respect to the total and PI positive cells respect to the total of pretreatment with TRAM-34 for 24 h in DOX (8 μM) induced feline kidney cell toxicity for 48 h, compared with DMSO control

Dose (μM)	Pretreatment 24 h					
	% of apoptotic cells respect to the total		t-test	% of PI positive cells respect to total		t-test
	TRAM-34 (n=3)	DMSO-control (n=3)	Significant (2-tailed)	TRAM-34 (n=3)	DMSO-control (n=3)	Significant (2-tailed)
0.1	15.263 ± 0.790	25.173 ± 1.256	0018*	5.949 ± 0.398	6.396 ± 0.292	0.629
0.5	15.168 ± 0.565	25.262 ± 1.089	0.009**	5.895 ± 0.581	6.375 ± 0.365	0.700
1	15.131 ± 0.557	26.650 ± 0.491	0.001***	6.028 ± 0.319	6.555 ± 1.503	0.853
5	27.690 ± 0.501	30.628 ± 1.355	0.306	7.003 ± 0.371	7.905 ± 0.863	0.608
10	31.473 ± 0.931	33.223 ± 0.823	0.462	10.037 ± 0.367	10.474 ± 0.472	0.694
25	40.780 ± 4.499	38.561 ± 1.709	0.803	18.623 ± 1.304	21.456 ± 1.620	0.475

h = hour

*Student's t-test is significant at p<0.05 level (2-tailed)

**Student's t-test is significant at p<0.01 level (2-tailed)

***Student's t-test is significant at p<0.001 level (2-tailed)

Table 16 Percentages of apoptotic cells respect to the total and PI positive cells respect to the total of pretreatment with TRAM-34 for 30 min in DOX (8 μM) induced feline kidney cell toxicity for 48 h, compared with DMSO control

Dose (μM)	Pretreatment 30 min					
	% of apoptotic cells respect to the total		t-test	% of PI positive cells respect to the total		t-test
	TRAM-34 (n=3)	DMSO-control (n=3)	Significant (2-tailed)	TRAM-34 (n=3)	DMSO-control (n=3)	Significant (2-tailed)
0.1	35.289 ± 0.744	33.747 ± 0.804	0.462	18.049 ± 0.924	18.542 ± 1.047	0.848
0.5	38.210 ± 1.897	33.834 ± 0.804	0.287	20.917 ± 0.848	19.256 ± 0.118	0.325
1	37.005 ± 2.031	35.404 ± 1.036	0.706	19.913 ± 0.479	20.094 ± 0.558	0.894
5	39.346 ± 1.253	40.669 ± 1.956	0.759	31.604 ± 2.838	31.676 ± 1.866	0.991
10	46.756 ± 0.864	49.724 ± 0.543	0.168	41.725 ± 1.831	40.058 ± 1.430	0.700
25	51.270 ± 0.204	52.604 ± 0.395	0.159	48.343 ± 0.570	51.561 ± 0.191	0.037*

*Student's t-test is significant at $p<0.05$ level (2-tailed)

4.2.1.3. Western blot analysis

According to cytotoxic assay, pretreatment with TRAM-34 at 0.1 to 1 μM concentration for 24 h protected feline kidney cells from DOX-induced cell toxicity. These concentrations of TRAM-34 were used to determine the KCa3.1 channel expression.

4.2.1.3.1. KCa3.1 protein expression

The KCa3.1 protein expression in pretreatment with TRAM-34 at concentrations of 0, 0.1, 1 and 10 μM in DOX-induced feline nephrotoxicity was shown (Table 17: Figure 17). The present study found the protein bands of KCa3.1 and β-actin which were 64 and 42 kDa, respectively (Figure 17A).

For pre-treatment 24 h, the DOX-treated control had significantly increased KCa3.1 protein expression than the negative control (p<0.05) (Table 17: Figure 17). Thus, DOX can induce feline kidney cells increased of KCa3.1 protein expression. Moreover, feline kidney cells of the pretreatment with TRAM-34 at 0.1 μM concentration (0.805 ± 0.052) had significantly decreased in KCNN4 protein expression when compared with DOX-treated control (1.413 ± 0.094) (p<0.05). On the other hand, KCa3.1 protein expression results showed no significant differences between DOX-treated control and cells treated with DMSO-control at 0.1 to 10 μM concentrations. (Table 17: Figure 17).

Table 17 KCa3.1 protein expression in pretreatment with TRAM-34 or DMSO-control at 0 to 10 μM concentrations for 24 h before DOX-induced feline kidney cell toxicity for 48 h

Pretreatment 24 h groups	KCa3.1 protein expression (Ratio of KCNN4/β-actin)	
	TRAM-34 (n=5)	DMSO-control (n=5)
Negative control	0.638 ± 0.049#	
DOX-treated control	1.413 ± 0.094*	
0.1 μM	0.805 ± 0.052#	1.399 ± 0.383
1 μM	1.032 ± 0.119	1.116 ± 0.186
10 μM	1.027 ± 0.104	1.106 ± 0.076*

h = hours, DOX = doxorubicin

*p < 0.05 when compared with negative control

#p < 0.05 when compared with DOX-treated control

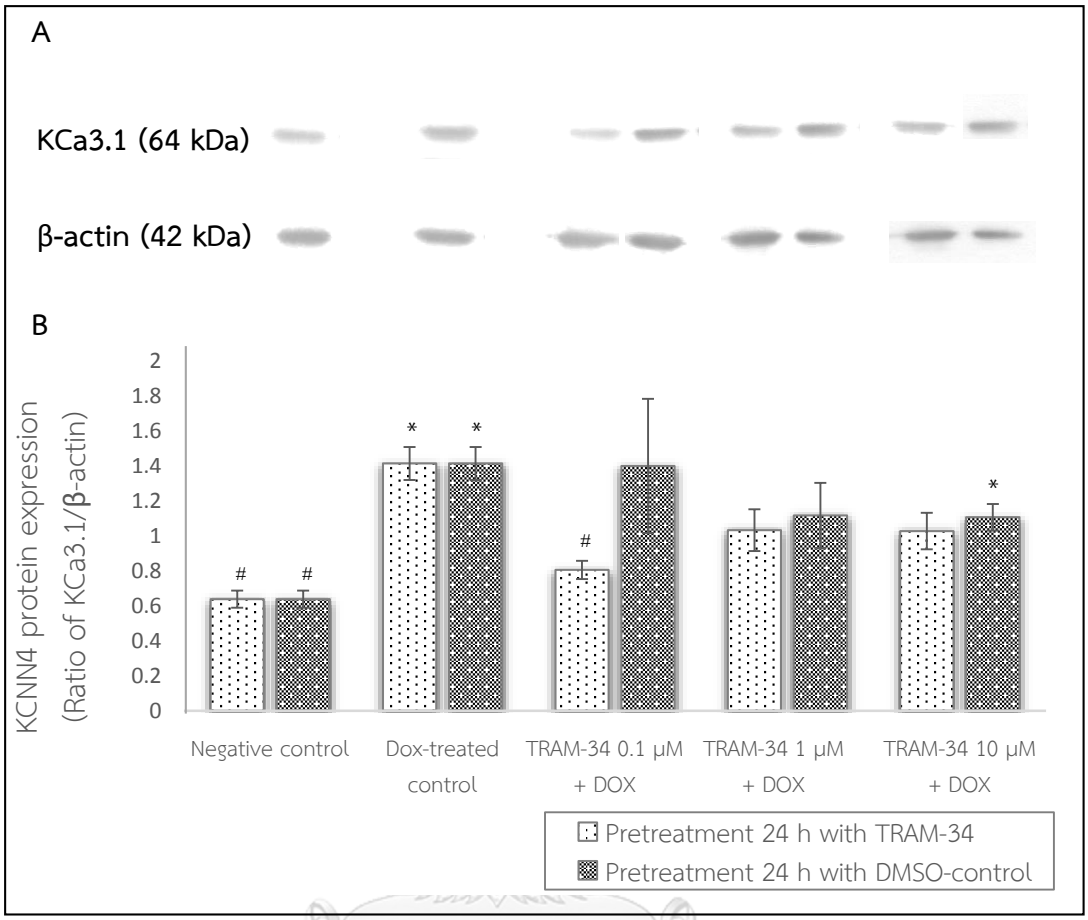


Figure 17 (A) KCa3.1 protein expression of pretreatment with TRAM-34 or DMSO-control at 0 to 10 μM concentrations for 24 h in DOX-induced feline kidney cell toxicity for 48 h. (B) Quantitative densitometry analysis of KCNN4 protein expression. *$p < 0.05$ when compared with negative control. #$p < 0.05$ when compared with DOX-treated control.

For pre-treatment 30 min, pretreatment with TRAM-34 at 0.1, 1 and 10 μM concentrations in DOX-induced feline kidney cell toxicity showed no significant difference in KCa3.1 protein expression when compared with DOX-treated control and the negative control (Table 18: Figure 18). However, KCa3.1 protein expression of cells treated with DMSO-control at 1 μM concentration had significantly higher than the negative control (p<0.05) (Table 18: Figure 18).

Table 18 KCa3.1 protein expression in pretreatment with TRAM-34 or DMSO-control at 0 to 10 μM concentrations for 30 min before DOX-induced feline kidney cells for 48 h

Pre-treatment 30 min groups	**KCa3.1 protein expression (Ratio of KCNN4/ β-actin)**	
	TRAM-34 (n=5)	**DMSO-control (n=5)**
Negative control	0.670 ± 0.017$^{\#}$	0.670 ± 0.017
DOX-treated control	1.267 ± 0.084*	1.267 ± 0.084
0.1 μM	1.186 ± 0.081	1.041 ± 0.138
1 μM	1.122 ± 0.127	1.083 ± 0.013*
10 μM	0.903 ± 0.093	1.203 ± 0.183

h = hours, DOX = doxorubicin

*p < 0.05 when compared with negative control

$^{\#}p$ < 0.05 when compared with DOX-treated control

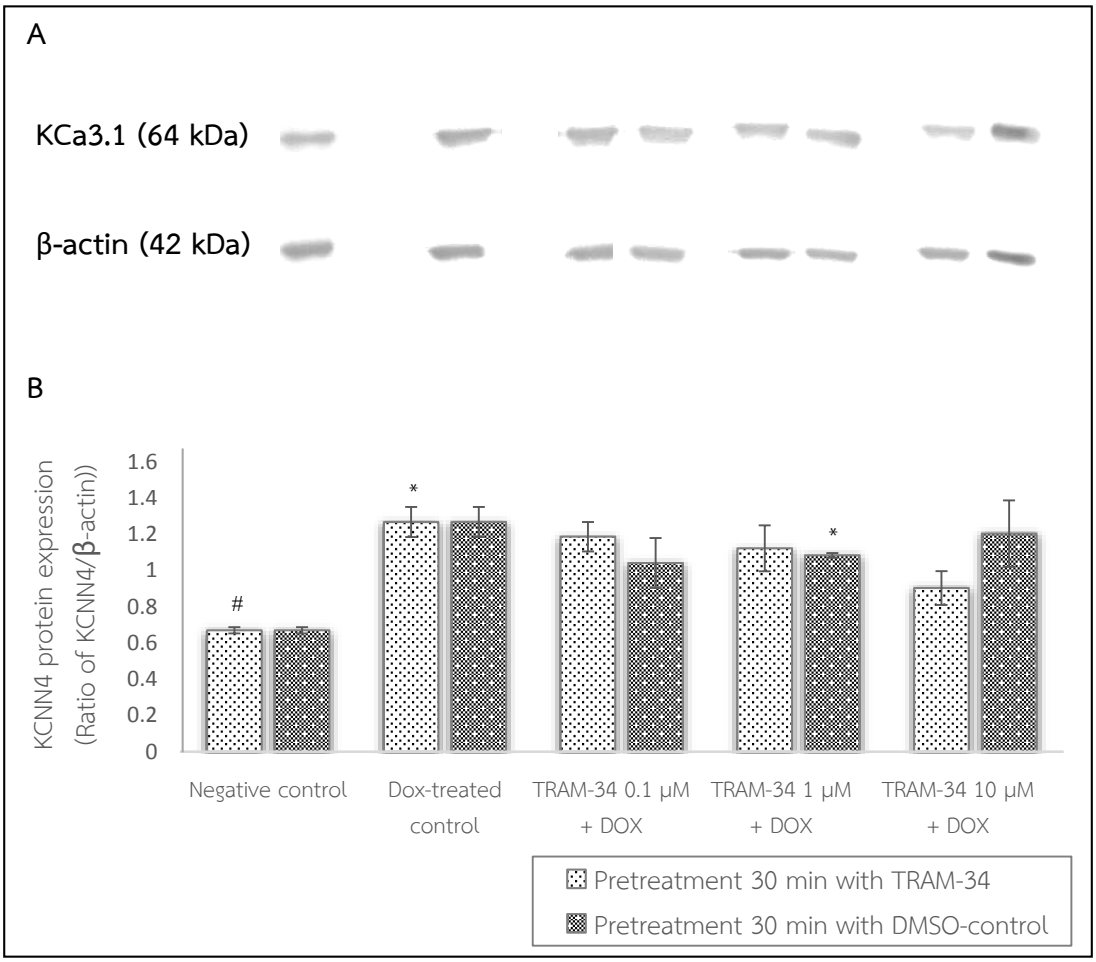


Figure 18 (A) KCa3.1 protein expression of pretreatment with TRAM-34 or DMSO-control at 0 to 10 μM concentrations for 30 min in DOX-induced feline kidney cell toxicity for 48 h. (B) Quantitative densitometry analysis of KCNN4 protein expression. *$p < 0.05$ when compared with negative control. $^{\#}p < 0.05$ when compared with DOX-treated control.

For compared between TRAM-34 and DMSO for each dose, KCa3.1 protein expression of pretreatment with TRAM-34 at 0.1, 1 and 10 μM concentrations for 24 h and 30 min was not significantly differences in KCa3.1 protein expression when compared with DMSO-control (p>0.05) (Table 19 and 20).

Table 19 KCa3.1 protein expression of pretreatment with TRAM-34 for 24 h in DOX (8 μM) induced feline kidney cell toxicity for 48 h, compared with DMSO control

Dose (μM)	Pretreatment 24 h		t-test
	TRAM-34 (n=5)	DMSO-control (n=5)	Significant (2-tailed)
0.1	0.805 ± 0.052	1.399 ± 0.383	0.196
1	1.032 ± 0.119	1.116 ± 0.186	0.715
10	1.027 ± 0.104	1.106 ± 0.076	0.556

Table 20 KCa3.1 protein expression of pretreatment with TRAM-34 for 30 min in DOX (8 μM) induced feline kidney cell toxicity for 48 h, compared with DMSO control

Dose (μM)	Pretreatment 30 min		t-test
	TRAM-34 (n=5)	DMSO-control (n=5)	Significant (2-tailed)
0.1	1.186 ± 0.081	1.041 ± 0.138	0.414
1	1.122 ± 0.127	1.083 ± 0.013	0.787
10	0.903 ± 0.093	1.203 ± 0.183	0.241

4.3. Feline kidney cells post-treatment with TRAM-34 after DOX treatment

Feline kidneys cell were treated for cell toxicity with 8 μM of DOX for 48 h. Then, the cells were treated with various concentrations of TRAM-34 for 24 h post DOX treatment. The treatment effects and KCa3.1 protein expression were determined in the experiment.

4.3.1. Treatment effects

The subtoxic doses of TRAM-34 were used to treated feline kidney cells after inducing nephrotoxicity with 8 μM of DOX for 48 h. The treatment effects, which included cytotoxicity assay, apoptosis and necrosis assay were measured.

4.3.1.1. Cytotoxicity assay

Feline kidney cells were treated with various concentrations of TRAM-34 (0, 0.1, 0.5, 1, 5, 10 and 25 μM) for 24 h after the induction of cell toxicity using 8 μM of DOX for 48 h. The results of post-treatment 24 h of TRAM at 0.1 to 25 μM concentrations showed significant cell death when compared with the negative control ($p<0.05$). In addition, percent cell viability of DOX-treated control was significantly difference with the negative control in post-treatment 24 h ($p<0.05$) (Table 21; Figure 19).

Table 21 Percent cell viabilities of post-treatment with TRAM-34 at 0 to 25 μM concentrations for 24 h in DOX (8 μM) induced feline kidney cell toxicity for 48 h

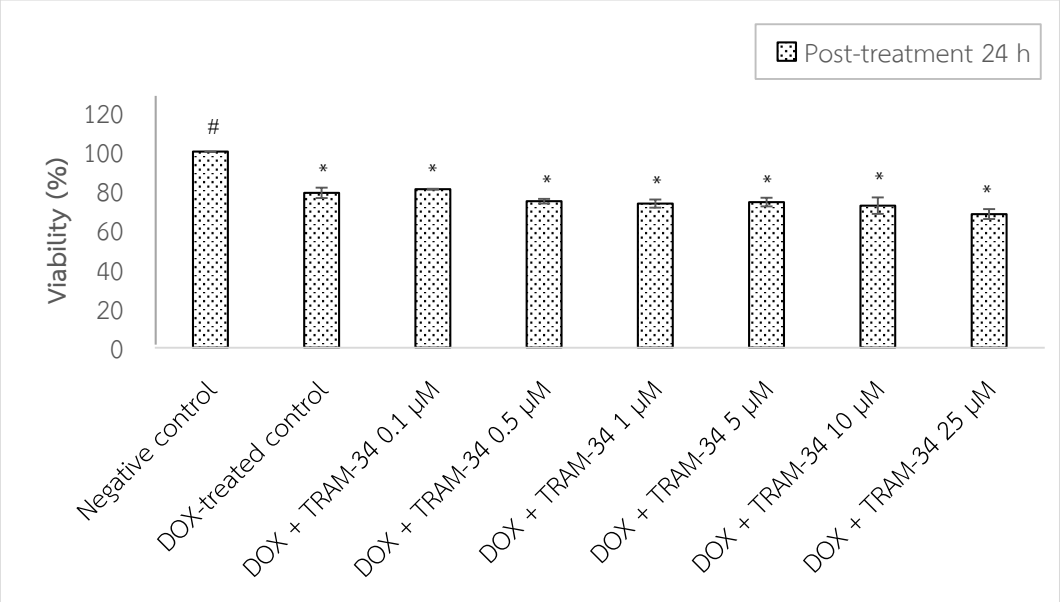
TRAM-34 (μM)	% cell viability Post-treatment 24 h (n=3)
Negative control	100[#]
DOX-treated control	78.965 ± 2.622*
0.1	80.843 ± 0.187*
0.5	74.696 ± 1.166*
1	73.487 ± 2.111*
5	74.260 ± 2.199*
10	72.449 ± 4.195*
25	68.129 ± 2.511*

DOX = doxorubicin, h = hour

*$p < 0.05$ when compared with negative control

[#]$p < 0.05$ when compared with DOX-treated control

Figure 19 Percent cell viabilities of post-treatment with TRAM-34 at 0 to 25 μM concentrations for 24 h in DOX (8 μM) induced feline kidney cell toxicity for 48 h



DOX = doxorubicin, h = hour

*$p < 0.05$ when compared with negative control

#$p < 0.05$ when compared with DOX-treated control

For DMSO control, post-treatment at 0.1, 0.5, 1 and 25 μM concentrations for 24 h had significantly lower percentage of cell viability than the negative control cells (p<0.05) (Table 22; Figure 20). Moreover, post-treatment with DMSO-treated control from 0.1 to 100 μM concentrations was not significantly differences in cell viability with DOX-treated control (p>0.05) (Table 22; Figure 20). Thus, the results showed no significant treatment effect of post-treatment 24 h with DMSO-treated control when compared with DOX-treated control.

Table 22 Percent cell viabilities of post-treatment with TRAM-34 or DMSO-control at 0 to 25 μM concentrations for 24 h in DOX (8 μM) induced feline kidney cell toxicity for 48 h

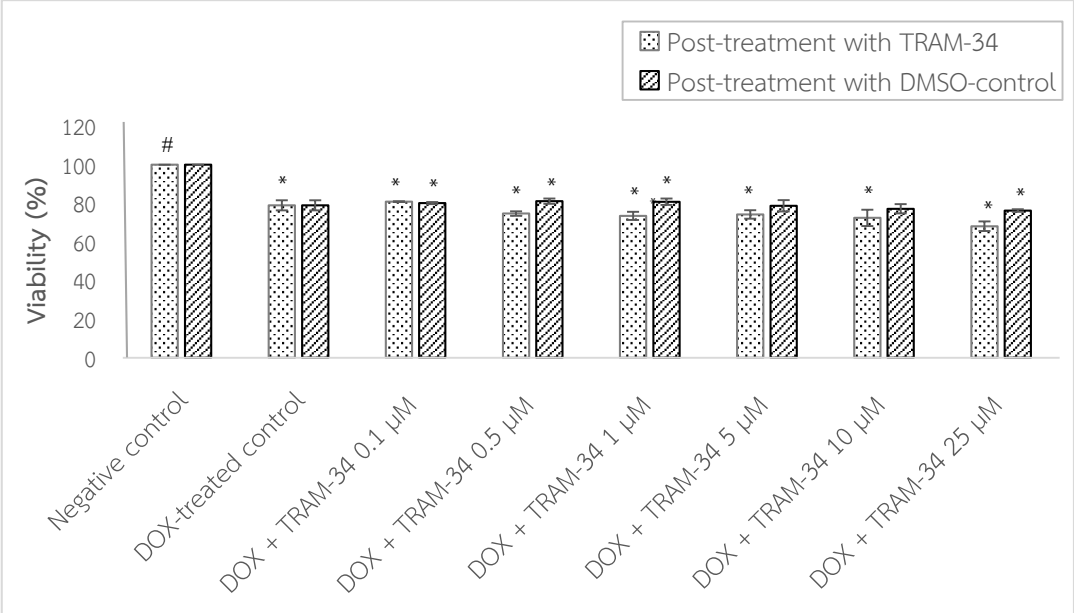
Post-treatment 24 h Groups	**% cell viability**	
	TRAM-34 (n=3)	**DMSO-control (n=3)**
Negative control	100[#]	100
DOX-treated control	78.965 ± 2.622*	78.965 ± 2.622
0.1 μM	80.843 ± 0.187*	80.096 ± 0.541*
0.5 μM	74.696 ± 1.166*	81.104 ± 1.376*
1 μM	73.487 ± 2.111*	80.818 ± 1.695*
5 μM	74.260 ± 2.199*	78.697 ± 2.957
10 μM	72.449 ± 4.195*	77.162 ± 2.379
25 μM	68.129 ± 2.511*	76.255 ± 0.642*

DOX = doxorubicin, h = hour

*p < 0.05 when compared with negative control

[#]p < 0.05 when compared with DOX-treated control

Figure 20 Percent cell viabilities of post-treatment with TRAM-34 or DMSO-control for 24 h in DOX (8 μM) induced feline kidney cell toxicity for 48 h



DOX = doxorubicin, h = hour

*$p < 0.05$ when compared with negative control

#$p < 0.05$ when compared with DOX-treated control

For compared between TRAM-34 and DMSO for each dose, Percent viability of post-treatment with TRAM-34 at 0.5 and 25 μM concentrations for 24 h was significantly lower than percent viability of DMSO-control (p<0.05) (Table 23). Post-treatment with TRAM-34 at 0.1, 1, 5 and 10 μM concentrations was not significantly differences in percent viability of cells when compared with DMSO-control.

Table 23 Percent (%) viability by MTT method of feline kidney cells at 0 to 100 μM concentrations of TRAM-34 treatment for 24 h, compared with DMSO control

Dose (μM)	Post-treatment 24 h		t-test
	TRAM-34 (n=3)	DMSO-control (n=3)	Significant (2-tailed)
0.1	80.843 ± 0.187	80.096 ± 0.541	0.262
0.5	74.696 ± 1.166	81.104 ± 1.376	0.024*
1	73.487 ± 2.111	80.818 ± 1.695	0.054
5	74.260 ± 2.199	78.697 ± 2.957	0.295
10	72.449 ± 4.195	77.162 ± 2.379	0.384
25	68.129 ± 2.511	76.255 ± 0.642	0.035*

*Student's t-test is significant at the 0.05 level (2-tailed)

4.3.1.2. Apoptosis and necrosis assay

The results of apoptosis and necrosis assay of post-treatment with TRAM-34 at 0 to 25 µg/ml concentrations for 24 h after the induction of cell toxicity using 8 µM of DOX for 48 h were listed (Table 24; Figure 21 and 22). The results of cell death from apoptosis were reported by percentage of apoptotic cells respect to the total and necrosis were reported by percentage of PI positive cells respect to the total.

The results of post-treatment for 24 h demonstrated significantly increased percentage of apoptotic cells respect to the total of post-treatment with TRAM-34 at 0, 5, 10, 25 and 50 µM concentration when compared with DOX-treated control (p<0.01). Percentage of apoptotic cells respect to the total of post-treatment with TRAM-34 at 0.1 to 50 µM concentration were significant higher than the negative control (p<0.01). Moreover, percentage of apoptotic cells respect to the total and percentage of PI positive cells respect to the total of DOX-treated control was significantly difference with the negative control in post-treatment 24 h (p<0.01) (Table 24; Figure 21 and 22).

For necrosis, the results of post-treatment with TRAM-34 at 0.5, 5, 10, 25 and 50 µM concentration were significant higher percentages of PI positive cells respect to the total than negative control (p<0.05). Likewise, post-treatment with TRAM-34 at 5 to 50 µM concentration had significantly increased percentages of PI positive cells respect to the total when compared with DOX-treated control (p<0.05) (Table 24; Figure 21 and 22).

Table 24 Percentage of apoptotic cells respect to the total and percentages of PI positive cells respect to the total of post-treatment with TRAM-34 at 0 to 25 μM concentrations for 24 h after DOX-induced feline kidney cells toxicity for 48 h

TRAM-34 post-treatment groups (μM)	Post-treatment 24 h	
	% of apoptotic cells respect to the total (n=3)	% of PI positive cells respect to the total (n=3)
Negative control	7.385 ± 0.304$^{\#\#}$	2.239 ± 0.107$^{\#\#}$
DOX-treated control	29.806 ± 1.912**	9.355 ± 0.249**
0.1	26.840 ± 0.745**	9.396 ± 0.639
0.5	29.901 ± 0.425**	9.576 ± 0.245**
1	30.937 ± 0.759**	13.972 ± 0.740
5	47.844 ± 2.075**$^{\#\#}$	33.176 ± 1.315*$^{\#}$
10	56.223 ± 0.921**$^{\#\#}$	43.343 ± 1.344*$^{\#}$
25	57.278 ± 1.774**$^{\#\#}$	43.621 ± 0.792 **$^{\#\#}$

DOX = Doxorubicin, h = hour

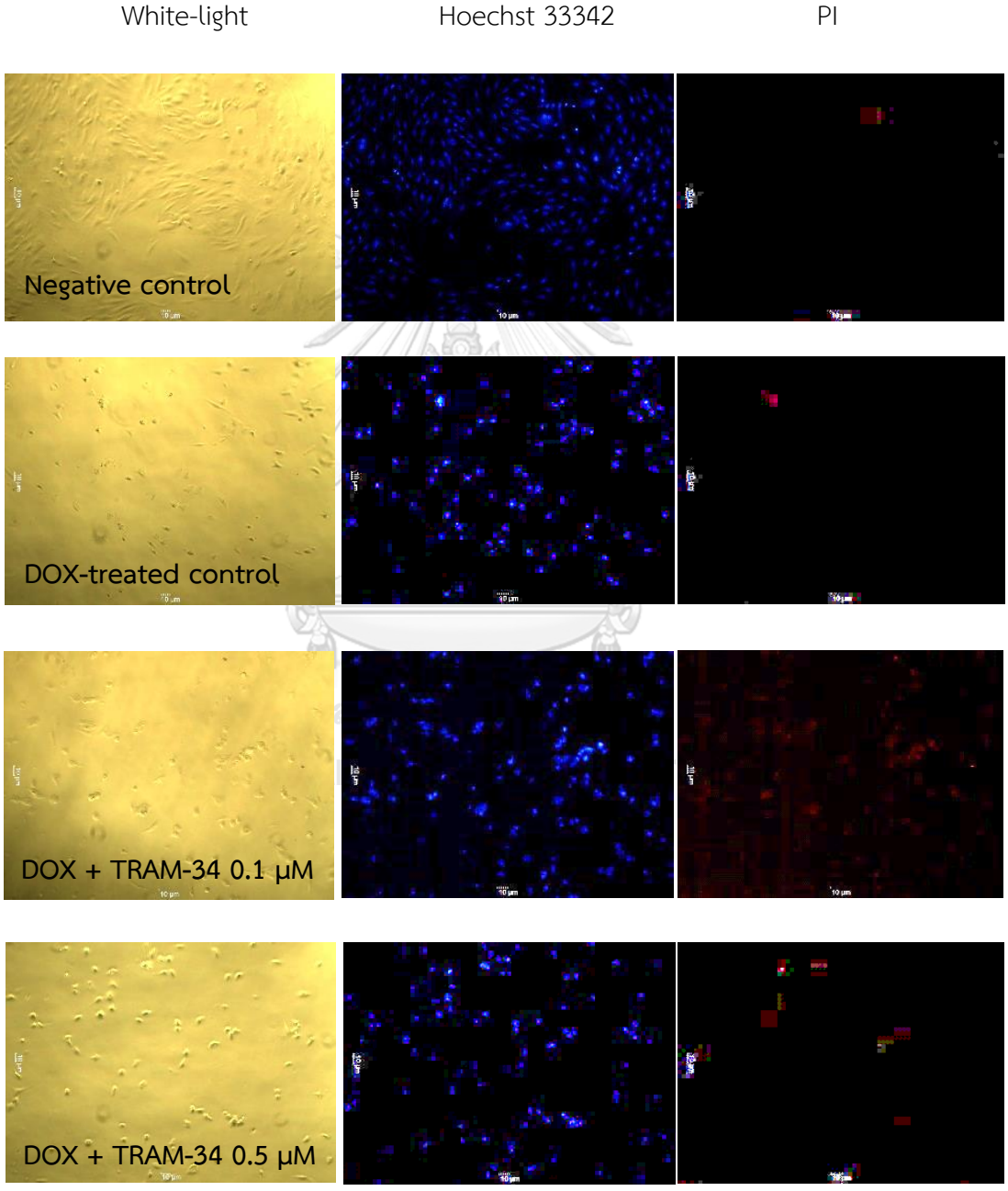
*p < 0.05 when compared with negative control

$^{\#}p$ < 0.05 when compared with DOX-treated control

**p < 0.01 when compared with negative control

$^{\#\#}p$ < 0.01 when compared with DOX-treated control

Figure 21 Feline kidney cells were induced cell toxicity with DOX for 48 h and then treated with TRAM-34 at 0 to 25 µM concentrations for 24 h. Apoptotic cells displayed cells with nuclear condensation stained in bright blue fluorescence and necrotic cells demonstrated damaged cells stained in red fluorescence.



White-light Hoechst 33342 PI

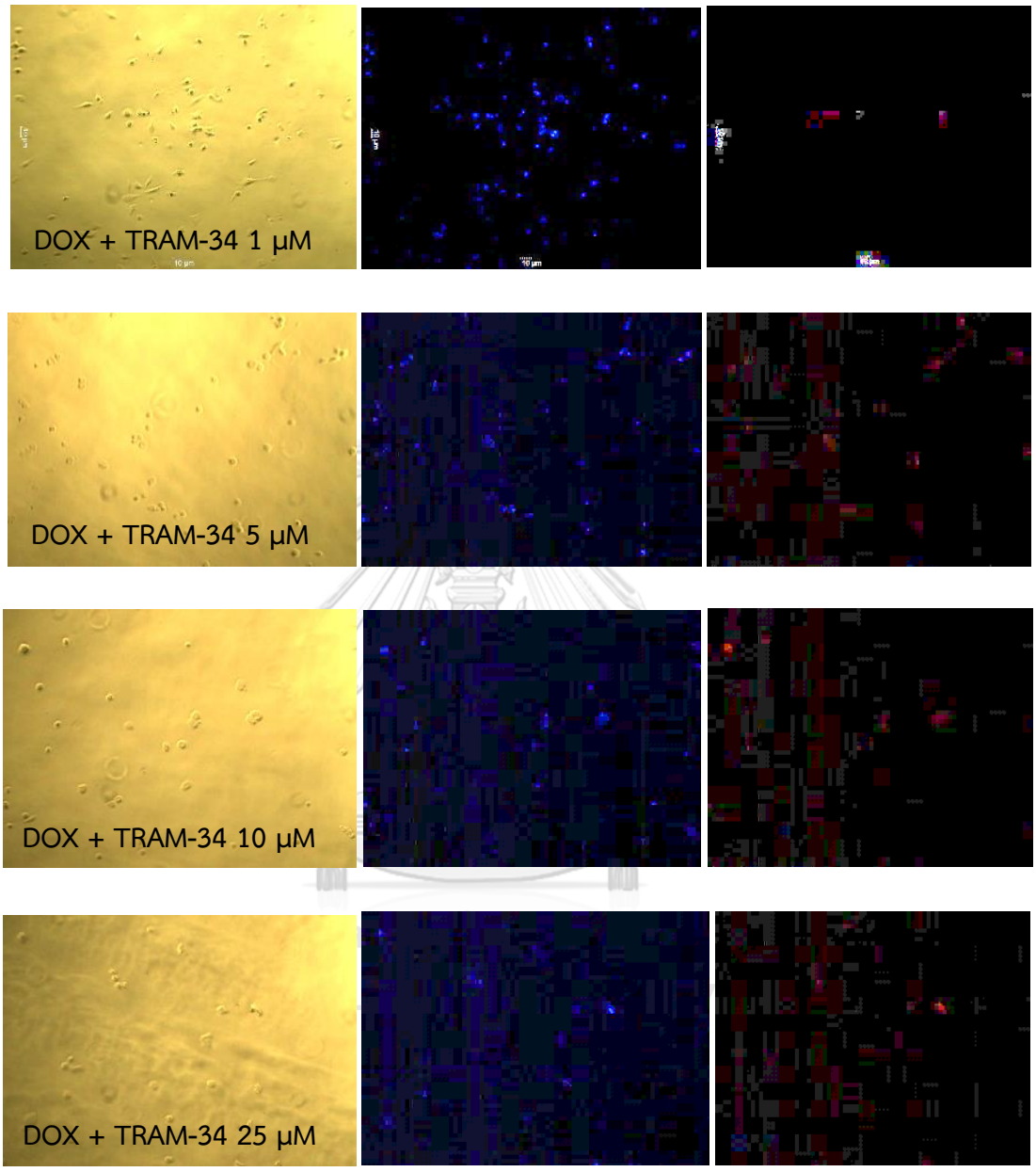
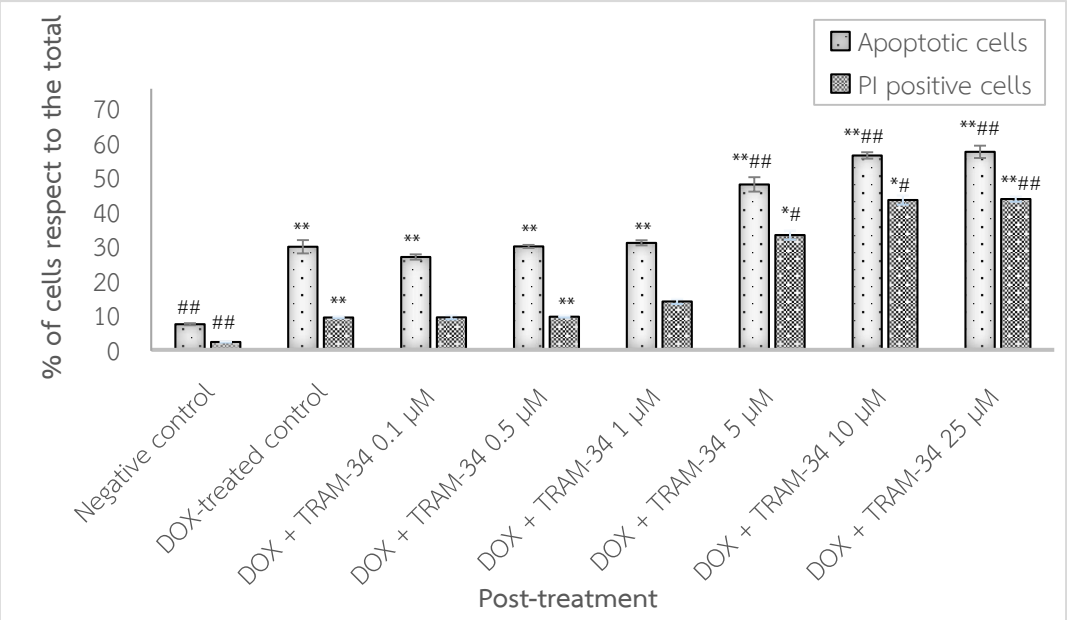


Figure 22 Percentages of apoptotic cells respect to the total and Percentages of PI positive cells respect to the total of feline kidney cells were induced cell toxicity with DOX for 48 h and then treated with TRAM-34 at 0 to 25 μM concentrations for 24 h.



DOX = Doxorubicin

*p < 0.05 when compared with negative control

#p < 0.05 when compared with DOX-treated control

**p < 0.01 when compared with negative control

##p < 0.01 when compared with DOX-treated control

For DMSO control, the results of post-treatment for 24 h with DMSO-treated control at 0.1 to 25 μM concentrations had significant higher percentage of apoptotic cells respect to the total than the negative control (p<0.05). Percentage of apoptotic cells respect to the total of DMSO-treated control at 25 μM concentrations had significantly increased cellular apoptosis when compared with DOX-treated control (p<0.05) (Table 25; Figure 23). For necrosis, the results of post-treatment for 24 h with DMSO-treated control at 0.1, 5, 10 and 25 μM concentrations had significant higher percentage of PI positive cells respect to the total than the negative control (p<0.05). In addition, DMSO-treated control at 5 to 25 μM concentrations had significant higher necrotic cells than DOX-treated control (p<0.05). Negative control had significantly lower percentage of PI positive cells respect to the total than DOX-treated control (p<0.01) (Table 25; Figure 23).

Table 25 Percentages of apoptotic cells respect to the total and Percentages of PI positive cells respect to the total of post-treatment with TRAM-34 or DMSO-control at 0 to 25 μM concentrations for 24 h after DOX-induced feline kidney cells toxicity for 48 h

TRAM-34 post-treatment groups (μM)	Post-treatment 24 h			
	% of apoptotic cells respect to the total		% of PI positive cells respect to the total	
	TRAM-34 (n=3)	DMSO-control (n=3)	TRAM-34 (n=3)	DMSO-control (n=3)
Negative control	7.385 ± 0.304$^{\#\#}$	7.385 ± 0.304	2.239 ± 0.107$^{\#\#}$	2.239 ± 0.107$^{\#\#}$
DOX-treated control	29.806 ± 1.912**	29.806 ± 1.912	9.355 ± 0.249**	9.355 ± 0.249**
0.1	26.840 ± 0.745**	30.027 ± 0.319**	9.396 ± 0.639	10.347 ± 0.489*
0.5	29.901 ± 0.425**	30.669 ± 0.374**	9.576 ± 0.245**	10.368 ± 1.179
1	30.937 ± 0.759**	33.415 ± 1.019*	13.972 ± 0.740	14.770 ± 2.195
5	47.844 ± 2.075**$^{\#\#}$	43.459 ± 1.298*	33.176 ± 1.315*$^{\#}$	28.552 ± 0.255**$^{\#\#}$
10	56.223 ± 0.921**$^{\#\#}$	54.086 ± 0.624**	43.343 ± 1.344*$^{\#}$	46.244 ± 1.695*$^{\#}$
25	57.278 ± 1.774**$^{\#\#}$	60.926 ± 0.997**$^{\#}$	43.621 ± 0.792**$^{\#\#}$	54.768 ± 1.007**$^{\#\#}$
50	64.181 ± 0.973^{ab}	65.700 ± 2.328*$^{\#}$	56.111 ± 1.897*$^{\#}$	56.437 ± 3.904

DOX = Doxorubicin, h = hour

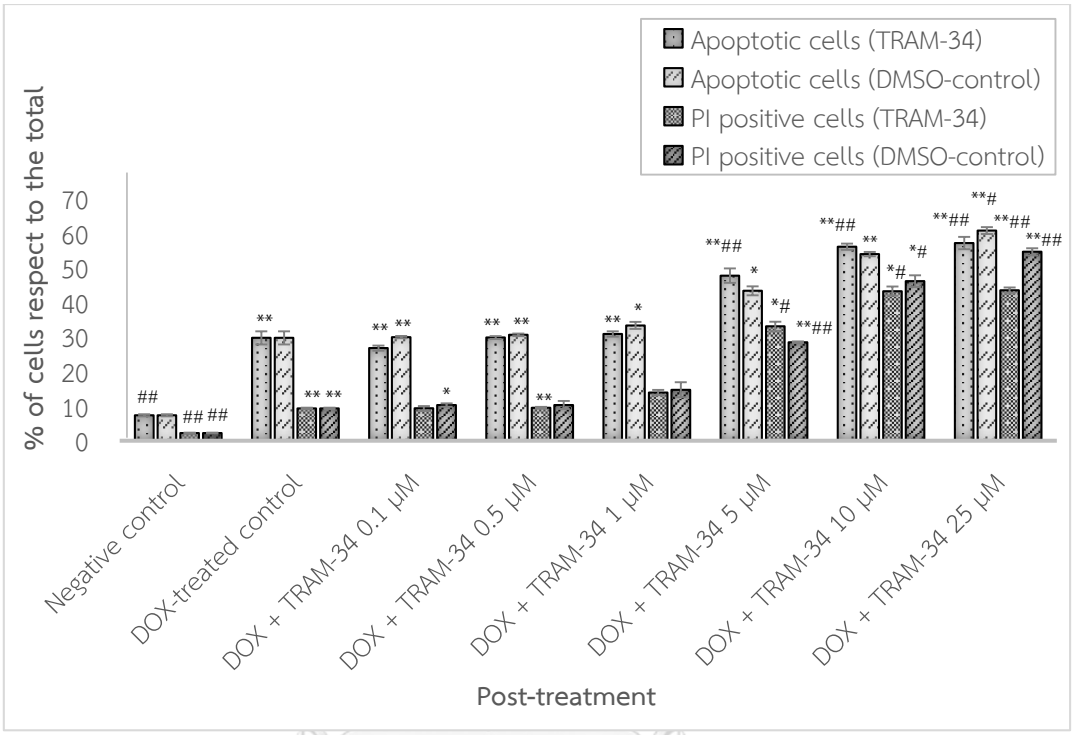
*p < 0.05 when compared with negative control

$^{\#}p$ < 0.05 when compared with DOX-treated control

**p < 0.01 when compared with negative control

$^{\#\#}p$ < 0.01 when compared with DOX-treated control

Figure 23 Percentages of apoptotic cells respect to the total and Percentages of PI positive cells respect to the total of post-treatment with TRAM-34 at 0 to 25 μM concentrations for 24 h after DOX-induced feline kidney cells toxicity for 48 h.



DOX = Doxorubicin, h = hour

*$p < 0.05$ when compared with negative control

#$p < 0.05$ when compared with DOX-treated control

**$p < 0.01$ when compared with negative control

##$p < 0.01$ when compared with DOX-treated control

For compared between TRAM-34 and DMSO for each dose, both percentages of apoptotic cells respect to the total and percentages of PI positive cells respect to the total of post-treatment with TRAM-34 at 0.1 to 25 μM concentrations for 24 h was not significantly differences when compared with DMSO-control ($p>0.05$), except percentages of PI positive cells respect to the total of TRAM-34 at 25 μM concentration for 30 min was significantly lower than DMSO-control ($p<0.01$) (Table 26).

Table 26 Percentages of apoptotic cells respect to the total and PI positive cells respect to the total of post-treatment with TRAM-34 at 0.1 to 25 μM concentrations for 24 h after DOX (8 μM) induced feline kidney cell toxicity for 48 h, compared with DMSO control

Dose (μM)	Post-treatment 24 h					
	% of apoptotic cells respect to the total		t-test	% of PI positive cells respect to total		t-test
	TRAM-34 (n=3)	DMSO-control (n=3)	Significant (2-tailed)	TRAM-34 (n=3)	DMSO-control (n=3)	Significant (2-tailed)
0.1	26.840 ± 0.745	30.027 ± 0.319	0.086	9.396 ± 0.639	10.347 ± 0.489	0.533
0.5	29.901 ± 0.425	30.669 ± 0.374	0.477	9.576 ± 0.245	10.368 ± 1.179	0.723
1	30.937 ± 0.759	33.415 ± 1.019	0.323	13.972 ± 0.740	14.770 ± 2.195	0.852
5	47.844 ± 2.075	43.459 ± 1.298	0.359	33.176 ± 1.315	28.552 ± 0.255	0.177
10	56.223 ± 0.921	54.086 ± 0.624	0.330	43.343 ± 1.344	46.244 ± 1.695	0.482
25	57.278 ± 1.774	60.926 ± 0.997	0.359	43.621 ± 0.792	54.768 ± 1.007	0.007**

**Student's t-test is significant at p<0.01 level (2-tailed)

4.3.1.3. Western blot analysis

4.3.1.3.1. KCa3.1 protein expression

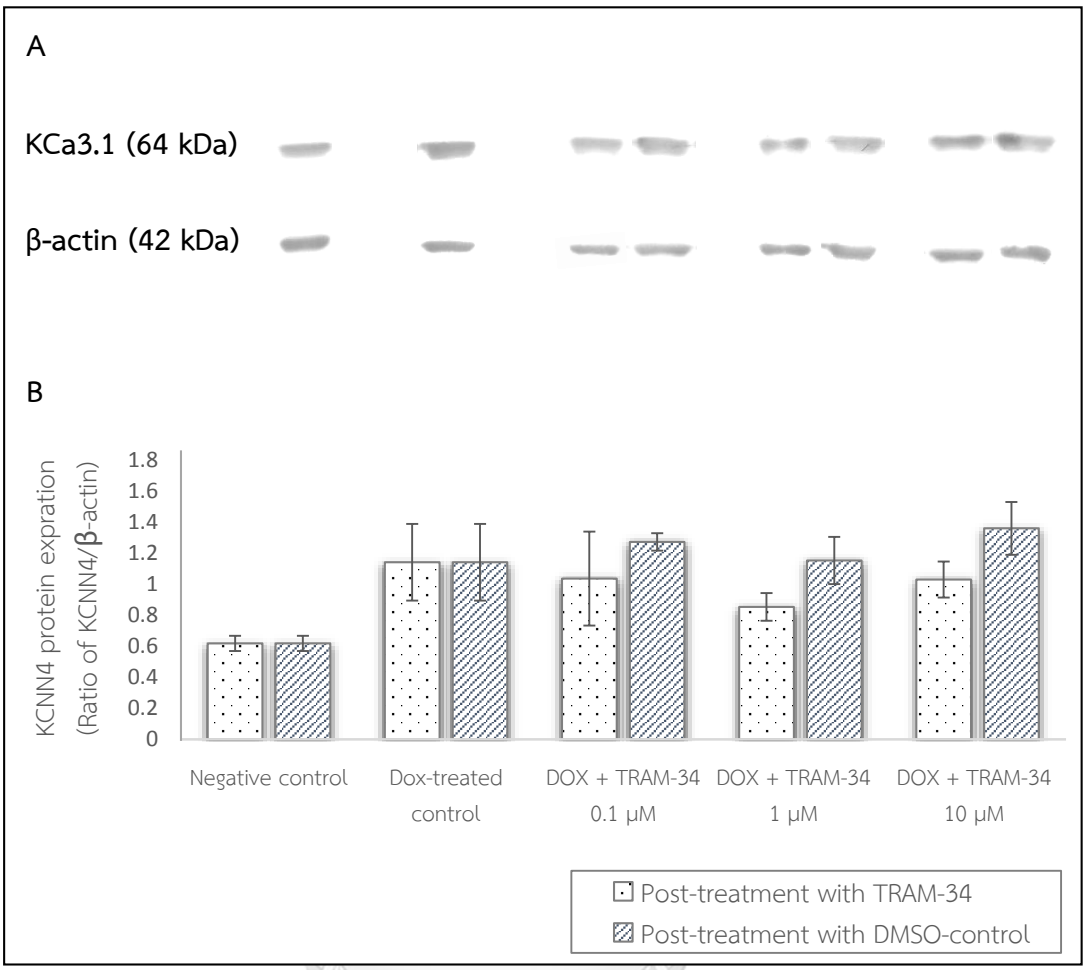
The KCa3.1 protein expression in post-treatment with TRAM-34 at concentrations of 0, 0.1, 1 and 10 μM concentrations in DOX-induced feline nephrotoxicity was shown (Table 27: Figure 24). Our study found the protein bands of KCa3.1 and β-actin which were 64 and 42 kDa, respectively (Figure 24A).

Post-treatment for 24 h with TRAM-34 or DMSO-control at 0.1, 1 and 10 μM concentrations in DOX-induced feline kidney cell toxicity for 48 h showed no significant difference in KCa3.1 protein expression when compared with the negative control and DOX-treated control (Table 27: Figure 24). Therefore, TRAM-34 post-treated 24 h had no treatment effect in feline kidney cells induced toxicity by DOX.

Table 27 KCa3.1 protein expression in post-treatment with TRAM-34 or DMSO-control at 0 to 10 μM concentrations for 24 h after DOX-induced feline kidney cell toxicity for 48 h

Post-treatment 24 h groups	KCa3.1 protein expression (Ratio of KCNN4/ β-actin)	
	TRAM-34 (n=3)	DMSO-control (n=3)
Negative control	0.618 ± 0.049	
DOX-treated control	1.140 ± 0.247	
0.1 μM	1.035 ± 0.302	1.271 ± 0.056
1 μM	0.853 ± 0.089	1.151 ± 0.152
10 μM	1.029 ± 0.116	1.358 ± 0.170

h = hours, DOX = doxorubicin



h = hours, DOX = doxorubicin

Figure 24 (A) KCa3.1 protein expression of post-treatment with TRAM-34 or DMSO-control at 0 to 10 μM concentrations for 24 h in DOX-induced feline kidney cell toxicity. (B) Quantitative densitometry analysis of KCNN4 protein expression.

For compared between TRAM-34 and DMSO for each dose, KCa3.1 protein expression of post-treatment with TRAM-34 at 0.1, 1 and 10 μM concentrations for 24 h was not significantly differences in KCa3.1 protein expression when compared with DMSO-control (p>0.05) (Table 28).

Table 28 KCa3.1 protein expression of pretreatment with TRAM-34 at 0.1 to 10 μM concentrations for 24 h in DOX (8 μM) induced feline kidney cell toxicity for 48 h, compared with DMSO control

Dose (μM)	Pretreatment 24 h		t-test
	TRAM-34 (n=5)	DMSO-control (n=5)	Significant (2-tailed)
0.1	1.035 ± 0.302	1.271 ± 0.056	0.485
1	0.853 ± 0.089	1.151 ± 0.152	0.103
10	1.029 ± 0.116	1.359 ± 0.170	0.184

CHAPTER V
DISCUSSION

5.1. Cytotoxicity of TRAM-34 in feline kidney cells

The results of the present study indicated that feline kidney cells treated TRAM-34 from 0.1 to 100 μM for 24 h, 50 μM for 48 h and 25 μM for 96 h were not toxic to feline kidney cells. However, the cell viability seem to be inversely relationship with dose of TRAM-34. Cell viability of feline kidney cells decreased when increased concentration of TRAM-34.

Even though, DMSO is widely used for dissolving drug in vitro testing and cryoprotective agent for cells biological studies (Adler et al., 2006; Yi et al., 2017). DMSO is a common vehicle of TRAM-34 in both in vivo and in vitro studies (Huang et al., 2013; Olivan-Viguera et al., 2013; Liu et al., 2015; Chen et al., 2016). Nevertheless, the results of the present study showed that feline kidney cells treated DMSO-control at 100 μM for 24 h and 48 h and 25 μM for 96 h had significantly lower percentage of cell viability than the negative control. These finding indicated that cell viability of feline kidney cells decreased when increased concentration and duration of TRAM-34 or DMSO. It might be speculated that DMSO with increased time and concentration especially in the final concentration at 1% was toxic to feline kidney cells. Previous studies have shown that DMSO reduced cell viability in a dose-dependent manner in human embryonic stem cells (Pal et al., 2012). The toxic effects also reported previously that ≥ 4% DMSO significantly caused apoptotic changes in PC12 cells (Wu et al., 2010), whereas cytotoxic effects were observed in EAhy926 cells with 0.6% DMSO (Yi et al., 2017). On the contrary, no changes in cell viability in up to 0.5% of DMSO concentration was demonstrated in embryonic stem cell line and 1% in other cell lines were also observed (Adler et al., 2006; Hebling et al., 2015). It was well recognized that the cells response to DMSO were depend on the dose and the type of cells.

Previous studies of TRAM-34 used DMSO as vehicle had no observable toxic effects to cells with DMSO less than 0.1% (Liu et al., 2015; Chen et al., 2016) and 0.5% final concentration added (Olivan-Viguera et al., 2013). Therefore, feline kidney cells treated with TRAM-34 from 0.1 to 25 μM concentrations (dissolved in DMSO less than

0.5% final concentration (v/v)) were not toxic to feline kidney cell lines in 96 h and could be used in feline kidney cells in the present study. Moreover, several studies noted that TRAM-34 itself has low toxicity and causes minimal cell death and apoptosis (Shepherd et al., 2007; Toyama et al., 2008). Wulff and Castle (2010) proposed that TRAM-34 at 10 μM concentration was non-toxic and did not inhibit cytochrome P450 activity. The recent study suggested that TRAM-34 has biologically active at the dose given, safe and well tolerated in human (Bertuccio and Devor, 2015). This study also has demonstrated that TRAM-34 is safe for used in feline kidney cell line.

5.2. Doxorubicin and KCa3.1 in feline kidney cells

In this study, DOX-treated control had significantly increased cell death and apoptotic when compared to the negative control in both pretreatment and post-treatment for 24 h concordant with previous study (Piyarungsri et al., 2014). DOX-induced cell apoptosis has been reported in several studies (Zhang et al., 1996; Chaotham et al., 2013). As in human renal proximal tubule cell death due to DOX through apoptosis pathway and necrotic cells were rarely observed (Chaotham et al., 2013). Similar to this study, the results were not significant different in percentage of necrotic cells between DOX-treated control and the negative control in pretreatment with TRAM-34 for 30 min and 24 h. It might indicate that DOX induced cell toxicity mainly by apoptosis mechanism. Studies reported that DOX-treated found the apoptosis of renal tubular cells in rats (Zhang et al., 1996) and human (Chaotham et al., 2013). Feline kidney cells has shown to have apoptosis mechanism though the use of DOX treated (Piyarungsri et al., 2014). Nevertheless, we also found that DOX-treated control had significantly increased the percentage of PI positive cells in post-treatment for 24 h when compared to the negative control as previously reported (Piyarungsri et al., 2014).

The present study reported that the KCa3.1 protein expression of pretreatment for 30 min and 24 h of feline kidney cells in DOX-treated control increased when compared with negative control. One study reported that KCa3.1 expression is increased in kidney cells and tissue of humans and mice with nephropathy (Huang et al., 2013) Thus, DOX might also induced kidney cell toxicity in feline kidney cells through KCa3.1 channel mechanisms causing an increase in KCa3.1 protein expression.

5.3. Cytotoxicity of pretreatment and post-treatment with TRAM-34 in DOX-induced feline kidney cells

Pretreatment for 24 h with TRAM-34 at 0.1 to 1 μM concentrations showed significantly decreased cell death in DOX-induced feline kidney cells and the percent cell viability was not different when compared with the negative control. In addition, cell viability of DMSO-control (0.1 to 100 μM) was not significantly differences with cell viability of DOX-treated control, which it had not protective effect in DMSO-control. These finding indicated that pretreatment with TRAM-34 at 0.1 to 1 μM concentrations for 24 h can protect cell death from DOX-induced cytotoxicity in feline kidney cell line. Several studies suggested that KCa3.1 involves in nephropathy and TRAM-34 can preserve renal cell damage (Bertuccio and Devor, 2015; Chen et al., 2016). Similar results were previously observed for pretreated with TRAM-34 (10 μM) can reduce cell death from cisplatin-induce cytotoxicity in human renal proximal tubular epithelial cells (HK-2 cells) (Chen et al., 2016). Biologically active of TRAM-34 appear at the potential doses given, which phamological blockade of KCa3.1 with TRAM-34 is dose-dependent (Grgic et al., 2009a; Bertuccio and Devor, 2015). TRAM-34 at 1 μM (SEM as ±1) concentration completely inhibited the KCa3.1 current (Olivan-Viguera et al., 2013).

Several studies added TRAM-34 after exposed cells to TGF-β1 (2 ng/ml) for inducing inflammation and fibrosis process be using TRAM-34 for 48 h at dose 4 μmol/L in HK2 cells (Huang et al., 2013), 2 μM in human primary renal fibroblast cells (Huang et al., 2014d) and 4 μM in HK2 cells (Huang et al., 2014a). All of these can attenuate cell damage, inflammation and fibrosis. Moreover, TRAM-34 (4 μM) for 6 days treatment can reduce cell injury by limited an increasing of CCL20 through inhibition of the NF-kB pathway in human proximal tubular cells were exposed to high glucose (Huang et al., 2014b). In mice model, several study reported TRAM-34 (peanut oil as vehicle) at dose 120 mg/kg/day was injected intraperitoneally and effectively blocked the activity of the KCa3.1 channel without exhibiting signs of toxicity (Toyama et al., 2008; Grgic et al., 2009a; Chen et al., 2016).

On the contrary, pretreatment for 30 min with TRAM-34 at 0.1 to 100 μM concentrations in feline kidney cells had significantly lower percent cell viability than DOX-treated control. These finding indicated that pretreatment with TRAM-34 at (0.1

to 100 μM) for 30 min had no protective effect from DOX-induced cytotoxicity in feline kidney cells. It was possible that 30 min time of pretreatment of TRAM-34 was not enough for the protective action. Whereas, previous study in the human proximal tubular cells pretreated with TRAM-34 (10 μM) for 30 min can protect cells from cisplatin-induce cytotoxicity (Chen et al., 2016). Pretreated with TRAM-34 (4 μM) for 2 h significantly reduced cell injury from TGF-β1 induced inflammation cytokine in HK-2 cells (Huang et al., 2014a).

A decrease in cell viability was observed in post-treatment with TRAM-34 (0.1 to 100 μM) when compared with the negative control. Cell viability of feline kidney cells decreased when increased dose of TRAM-34. Cell viability of DMSO-control (0.1 to 100 μM) was not significantly differences from DOX-treated control in post-treatment for 24 h. These results indicated that post-treatment with TRAM-34 (0.1 to 100 μM) for 24 h demonstrated no protective effect from DOX-induced cytotoxicity in feline kidney cells. Interestingly, no previous studies investigated the effect of post-treatment of TRAM-34 in kidney cells.

5.4. Apoptosis and necrosis of pretreatment and post-treatment with TRAM-34 in DOX-induced feline kidney cells

Pretreatment with TRAM-34 at 0.1 to 1 μM concentrations for 24 h had significantly decreased apoptotic cells when compared to DOX-treated control. Pretreatment with TRAM-34 (0.1 to 1 μM) can protect apoptosis from DOX-induced kidney cell toxicity in feline kidney cell line. Similar results of TRAM-34 (10 μM) can protect cells apoptosis in human renal proximal tubular cells, development of renal injury and apoptosis in mice (Chen et al., 2016). TRAM-34 can protect apoptosis in kidney cells by inhibiting the KCa3.1 activities. KCa3.1 channel might play a role in apoptosis through calcium signaling pathway in cellular processes (Bradding and Wulff, 2009; Wulff and Castle, 2010; Shao et al., 2011). Other activity of potassium channels has been related to apoptosis such as excessive potassium efflux promoted central neuronal apoptosis (Chen et al., 2013). However, type of potassium channel in response to different apoptosis-inducing stimuli is still unknown (Chen et al., 2013). KCa3.1 is activated, results in intracellular potassium efflux that participates in the regulation of cell shrinkage and apoptotic volume decrease and apoptosis (Remillard

and Yuan, 2004) such as erythrocyte and lymphocyte apoptosis (Elliott and Higgins, 2003; Lang et al., 2004). KCa3.1 channel as mediators of intrinsic and extrinsic apoptotic cell death by regulated cell condensation known as apoptotic volume decrease. KCa3.1 is also involve in intrinsic apoptotic pathway in the staurosporine-induced glioblastoma cells using TRAM-34 can reduce caspase-3 activation (McFerrin et al., 2012). Similar to cisplatin-induced HK-2 cells apoptosis attenuation by TRAM-34 through interference with intrinsic apoptotic (caspase-3, Bax, Bak, caspase-9) and endoplasmic reticulum stress-related (caspase-12) mediators and may suppress calcium-related signaling (Chen et al., 2016).

On the other hand, the results of pretreatment for 30 min demonstrated TRAM-34 (0.1, 10 and 25 μM) was significant higher in apoptotic cells than negative control and had no different with DOX-treated control. These results indicated that TRAM-34 could not reduce apoptosis cells from DOX-induced cell toxicity in feline kidney cell line. Whereas, the previous study reported that the cells pretreated with TRAM-34 (10 μM) for 30 min inhibited cell apoptosis from cisplatin-induce cytotoxicity in human proximal tubular cells (Chen et al., 2016). It was possible that duration or time of pretreatment with TRAM-34 was significantly associate with the protective effect of TRAM-34 from nephropathy.

Post-treatment for 24 h with TRAM-34 had significantly higher percentage of apoptotic cells than DOX-treated control and the negative control. Consequently, there was no treatment effect of TRAM-34 in cell apoptosis from DOX-induced cell toxicity in feline kidney cell line. To author's knowledge, there was no study about apoptosis of cell in post-treatment with TRAM-34 in kidney cells.

For the effects of TRAM-34 on the necrosis of cells, both pretreatment with TRAM-34 for 30 min and 24 h were not different in percentage of PI positive cells with DOX-treated control. Furthermore, post-treatment with TRAM-34 had significant increasing of necrotic cells when compared with negative control and DOX-treated control. These results displayed that TRAM-34 post treated after DOX could not inhibit cell necrosis from DOX-induced cell toxicity in feline kidney cell line.

5.5. KCa3.1 protein expression of pretreatment and post-treatment with TRAM-34 in DOX-induced feline kidney cells

In this study, the results showed that bands at 64 kDa were significantly detected and demonstrated that KCa3.1 proteins (64 kDa) appeared at higher expected bands (48 kDa). It may be due to post-translational modifications. However, the KCNN4 primary antibody (rabbit polyclonal to KCNN4) used in this study was recommended for detecting rat, mouse and human KCNN4 or KCa3.1 channel. It was possible that cells form different species may respond differently to antibodies. However, by blast performing the sequence homology, the immunogen of polyclonal KCa3.1 antibody (ab215990, Abcam) shares 94.4 % homology with the cat protein (potassium calcium-activated channel subfamily N member 4; KCNN4 of *Felis catus*). It was predicted that this antibody might cross-reaction with cat samples. According to the study of Morales et al. (2013), it has been reported that the band of KCa3.1 linked to CaM (KCa3.1-CaM) displayed at 57 kDa, KCa3.1 monomer at 40 kDa and a band at 17 kDa corresponding to CAM (Morales et al., 2013). It was possibly that there was an association of KCa3.1 and CaM in cat samples from feline kidney cells to increasing molecular weight of KCa3.1. Most studies reported that CaM is mainly protein for gating process of KCa3.1 channel (Mene and Pirozzi, 2010; Morales et al., 2013)

The present study reported that pretreatment with TRAM-34 at 0.1 μM for 24 h to feline kidney cells had significantly lower in KCa3.1 protein expression than DOX-treated control, but not statistically significant from DMSO-control. Moreover, KCa3.1 protein expression of feline kidney cells in DOX-treated control significantly increased when compared with negative control. These results indicated that TRAM-34 (0.1 μM) reduced KCa3.1 protein expression on DOX-induced cytotoxicity in feline kidney cells. There were a rise in KCa3.1 protein expression in feline kidney cells with DOX-induced nephropathy. Similarly, studies reported that KCa3.1 protein expression were increased in damaged renal cells both in vivo and vitro (Grgic et al., 2009a; Chen et al., 2016). Grgic et al. (2009) demonstrated that there was an increased in KCa3.1 protein expression in mice with induced UUO (as a progressive renal fibrosis model) and TRAM-34 can attenuate the progression of renal fibrosis in UUO-induced mice. Similar to the in vitro finding, there was up-regulation of KCa3.1 protein expression in mitogenically

stimulated murine renal fibroblast (Grgic et al., 2009a). In human, KCa3.1 protein expression elevated in cisplatin-induced renal cell injury in HK2-cells (Chen et al., 2016). Similar to the in vivo finding, mice with renal tubular damage by cisplatin displayed an increasing of KCa3.1 protein expression (Chen et al., 2016). On the contrary, TRAM-34 can reduce KCa3.1 protein expression and renal cell damage from cisplatin-induced cytotoxicity in HK-2 cells and mice with cisplatin-induced kidney injury (Chen et al., 2016). It might be speculated that an increasing of KCa3.1 protein expression is related to nephrotoxicity and apoptosis in the kidney cells.

The expression of KCa3.1 protein of both pretreatment for 30 min and post-treatment for 24 h with TRAM-34 (0.1 to 10 μM) was not different from DOX-treated control. Thus, the KCa3.1 expression results correlated with the results of cell viability assay and apoptosis and necrosis assay in both pretreatment and post-treatment of TRAM-34 on feline kidney cell line.

Pretreatment with TRAM-34 at 0.1 μM concentration for 24 h showed significant protective effect from DOX-treated on feline kidney cells. On the other hand, TRAM-34 given to feline kidney cells after the induction of nephrototoxicity by DOX, at 0.1 to 25 μM concentrations could not protect feline kidney cells. This results indicated that TRAM-34 post-treatment had no treatment effect. Thus, TRAM-34 must be used as pretreatment before cats develop nephropathy.

In conclusion, TRAM-34 (0.1 to 25 μM) were not toxic to feline kidney cell lines in 96 h. In addition, pretreatment with TRAM-34 (0.1 μM) could protect feline kidney cells from DOX-induced cell toxicity. TRAM-34 may be the potential renal protective therapeutic in cats with naturally occurring CKD in the future and need to be further evaluated in the in vivo model.

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