APPENDICES

APPENDIX A

Research article

Differential chemosensitisation of P-glycoprotein overexpressing K562/Adr cells by Withaferin A and Siamois polyphenols

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Running title: Selective cancer chemosensitisation by different NFκB inhibitors

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Abbreviations: NFκB, nuclear factor κB; IκB, inhibitory subunit of NFκB; IKK, IκB kinase; TNF, tumour necrosis factor; IL, interleukin; EMSA, electrophoretic mobility shift assay

Abstract

Multidrug resistance (MDR) is a major obstacle in cancer treatment and is often the result of overexpression of the drug efflux protein, P-glycoprotein (P-gp) as a consequence of hyperactivation of NFκB, AP1 and Nrf2. In addition to effluxing chemotherapeutic drugs, P-gp also plays a specific role in blocking caspase-dependent apoptotic pathways. One feature that cytotoxic treatments of cancer have in common is activation of the transcription factor NFκB, which regulates inflammation, cell survival and P-gp expression and suppresses the apoptotic potential of chemotherapeutic agents. As such, NFκB inhibitors may promote apoptosis in cancer cells and could be used to overcome resistance to chemotherapeutic agents.

Interestingly, we found that doxorubicin-sensitive K562 and -resistant K562/Adr cells differentially express NFκB target genes involved in inflammation, angiogenesis, cell cycle, metastasis, anti-apoptosis and MDR. Furthermore, we demonstrate that different classes of NFκB inhibitors, i.e. the withasteroid Withaferin A and Siamois polyphenols (quercetin, eriodictyol) similarly inhibit NFκB target gene expression and elicit induction of early apoptosis in K562 and K562/Adr cells. Surprisingly, only Withaferin A but none of the Siamois polyphenols is able to trigger late apoptosis and elicit caspase-3 activation in K562/Adr cells. This demonstrates that chemoresistance in P-gp overexpressing cells with impaired caspase activation and late apoptosis can only beovercome by specific classes of NFκB inhibitors.

Introduction

The cytotoxicity of chemotherapeutic agents is attributed to apoptosis. One feature that cytotoxic treatments of cancer have in common is their activation of the transcription factor NFκB, which regulates cell survival, suppresses the apoptotic potential of chemotherapeutic agents and contributes to resistance (Nakanishi & Toi, 2005). Acquired resistance to the effects of chemotherapy has emerged as a significant impediment to effective cancer therapy. As such it is believed that inhibitors of NFκB might promote apoptosis in cancer cells and can be used to overcome resistance to chemotherapeutic agents.

Nuclear factor kappa B (NFκB) is a family of transcription factors that play important roles in regulating cell differentiation, proliferation, immune response and blocking apoptosis (Hayden & Ghosh, 2004; Schmitz et al, 2004). In mammalian cells, the NFκB/Rel family consists of five members: RelA (p65), RelB, c-Rel, p105/p50 (NFκB1), and p100/p52 (NFκB2). Each family member has a conserved Rel homology domain specifying DNA binding, protein dimerization, and nuclear localization. In most cells, NFκB is composed of a heterodimer of p65 and p50, where the p65 protein is responsible for the transactivation potential. In unstimulated cells, NFκB is sequestered predominantly in the cytoplasm in an inactive complex through interaction with IκB inhibitor proteins. In response to stimulation by a variety of potent activators, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1, phorbol ester (PMA) or lipopolysaccharide (Wei et al, 2003) and cytotoxic drug ??, IκBα is rapidly phosphorylated at two conserved NH_2-terminal serines (Ser-32 and Ser-36) and degraded through a ubiquitin-dependent proteolysis, resulting in the release of NFκB, translocation into the nucleus, and induction of gene transcription. The NFκB has a role in oncogenesis and regulation of cancer therapy sensitivity. Overexpression, amplification, and rearrangements of different genes related to NFκB have been observed in tumors (Rayet & Gelinas, 1999). NFκB is activated in response to various inflammatory stimuli including cytokines, mitogens, bacterial products, viral proteins, and apoptosis-inducing agents (Baeuerle & Baichwal, 1997; Karin & Greten, 2005). Constitutive expression of NFκB leads to activation of several factors involved in cell cycle progression and cell differentiation for cancer metastasis. Inhibiting NFκB activity in tumor cells dramatically

reduces cell growth *in vitro* and *in vivo* (Duffey et al, 1999). NFκB, possibly through the activation of the antiapoptotic genes, plays a key role in the protection of cells against inducers of apoptosis including chemotherapeutic drugs (Barkett & Gilmore, 1999). Several mechanisms including increased expression of NFκB proteins, mutations and/or deletions in IκBa gene, and increased IκBa turnover, are involved in NFκB hyperactivation in tumor cells (Krappmann et al, 1999; Rayet & Gelinas, 1999). As such, various therapeutic strategies aim to decrease chronic hyperactivated NF-κB by pharmacological as well as phytomedicinal approaches in cancer (Aggarwal et al, 2009; Baud & Karin, 2009; Gong et al, 2003; Surh, 2003; Vanden Berghe et al, 2006a). NF-κB-regulated genes are involved in cell death, invasiveness, proliferation, angiogenesis, inflammation and MDR. One of the most important mechanisms by which tumor cells resist cytotoxic effects of a variety of chemotherapeutic drugs (including vinblastine, doxorubicine, etoposide and teniposide as well as many other cytotoxic agents) is overexpression of the *mdr1* gene and its product, P-glycoprotein (P-gp) (Gottesman et al, 2002).

P-gp is 180 kDa protein belonging to the ATP-binding cassette (ABC) superfamily of membrane transporter proteins (Biedler, 1994; Bosch & Croop, 1996). It is expressed in various tissues such as kidney tubules, colon, pancreas and adrenal gland, and tumors derived from these tissues are often resistant to chemotherapeutic drugs. Furthermore, *mdr1* expression is also increased in many relapsing cancers. P-gp is an energy-dependent drug efflux pump that maintains intracellular drug concentrations below cytotoxic levels, thereby decreasing the cytotoxic effects of a variety of chemotherapeutic agents, including anthracyclines, vinca alkaloids, and epipodophyllotoxins (Goldstein et al, 1989; Gottesman et al, 2002). P-gp also plays a role in inhibition of drug accumulation and caspase activation in the MDR tumor (Friedrich et al, 2001; Johnstone, 2002; Ruefli et al, 2002). Of special note, NFκB-mediated drug resistance was found to depend on the regulation of P-gp (Bentires-Alj et al, 2003). In addition, NFκB-dependent regulation of P-gp expression has also been demonstrated in renal tubules or liver (Ros et al, 2001; Thevenod et al, 2000). By upregulation of P-gp expression, NFκB was found to control drug efflux in cancer cells.

Cancer cells contain multiple signal transduction pathways of which theactivities are frequently elevated due to their transformation, and that are often activated following exposure to established cytotoxic therapies including ionizing radiation and chemical DNA damaging agents. Many pathways activated in response to transformation or toxic stresses promote cell growth and invasion and counteract the processes of cell death. As a result of these findings many drugs with varying specificities, have been developed to block signaling by cell survival pathways in the hope of killing tumor cells and sensitizing them to toxic therapies. Unfortunately, due to the plasticity of signaling processes within a tumor cell, inhibition of any one growth factor receptor or signaling pathway frequently has only modest long-term effects on cancer cell viability, tumor growth, and patient survival. As a result of thisobservation, a greater emphasis has begun to be puton multifocal natural compounds, such as polyphenols, withanolides, xanthones, indanones, curcuminoids, which simultaneously inhibit multiple inter-linked signal transduction/survival pathways (Bracke et al, 2008; Kaileh et al, 2007; Kunnumakkara et al, 2008; Obolskiy et al, 2009; Pinto et al, 2005; Surh, 2003). This, it is hoped, will limit the ability of tumor cells to adapt and survive because the activity within multiple parallel survival signaling pathways has been reduced (Paul et al, 2006). As such, over the past decades, researchers searching for new drugs to use in oncology have refosuced on natural products (Nakanishi & Toi, 2005; Paul et al, 2006).

As modern medicine continues to expand, so do the uses of phytomedicinal preparations. Polyphenols or phenolic compounds encompass molecules that possess an aromatic ring bearing one or more hydroxyl substituents. Natural polyphenols can range from simple molecules, such as phenolic acid, flavonoids and large highly polymerized compounds, such as tannins (Harborne & Williams, 2000). This class of phytochemicals can be found in high concentrations in wide varieties of higher plants and their products, such as wine and tea. They were also demonstrated to exert a wide range of biological activities including antioxidant, anticarcinogenic, antiproliferative, antimicrobial anti-inflamatory and apoptosis-inducing actions (Choi et al, 2001; Csokay et al, 1997; Formica & Regelson, 1995; Jeyabal et al, 2005; Terra et al, 2007).

Various polyphenols have been characterized with respect to their anti-invasive potential. Because invasion is, either directly or via metastasis formation, the main cause of death in cancer patients, development of efficient anti-invasive agents is an important research challenge (Bracke et al, 2008). Vanden Berghe *et al*. showed that

phytoestrogenic soy isoflavones can selectively block nuclear NFκB transactivation of specific NFκB target genes independently of their estrogenic activity in highly metastatic breast cancer cells (Vanden Berghe et al, 2006a). In 12-O-tetradecanoylphorbol-13-acetate (TPA) induced mouse skin tumor, the oligomeric and polymeric polyphenols decreased TPA-induced cell proliferation by attenuating activation of signalling kinases [c-Jun N-terminal protein kinase (JNK), extracellular signal-regulated protein kinase-1/2 (ERK1/2), p38 protein kinase and Akt], transcription factors [activator protein-1 (AP1) and NFκB] and inflammatory protein [cyclooxygenase-2 (Cox-2)] (Kundu et al, 2009; Patel et al, 2008). The NFκB and Akt kinase pathway, which play critical roles in inflammation, vascular homeostasis and angiogenesis, were repressed by the polyphenolic compound deguelin in human vascular endothelial cells and *HT1080* fibrosarcoma cells and chronic lymphocytic leukemia cells (Dell'Eva et al, 2007; Geeraerts et al, 2007; Matsuda et al, 2007). Nitric oxide production was reduced by the green tea polyphenols (-)-Epigallocatechin-3-gallate (EGCG) and black tea theaflavins by suppressing inducible nitric oxide synthase in a breast cancer cell line (Beltz et al, 2006). The latter treatment blocks nuclear translocation of the transcription factor NFκB as a result of decreased IkappaB kinase activity. However, anti-cancer effects of polyphenols may indirectly also involve effects on immune cells at the cancer-inflammation interface. Several studies demonstrated that polyphenolic compouds exhibit antiinflammatory activity in activated macrophages by inhibiting NFκB signaling pathway (Hamalainen et al, 2007; Lin et al, 1999; Terra et al, 2007). Dijsselbloem *et al*. demonstrated that genistein inhibits IL6 gene expression by modulating the transcription factor NFκB in TLR4-stimulated dendritic cells (Dijsselbloem et al, 2007). Pycnogenol inhibits TNFα-induced NFκB activation and adhesion molecule expression in human vascular endothelial cells (Peng et al, 2000). Red wine polyphenols, delphinidin and cyanidin inhibit platelet derived growth factor$_{AB}$ (PDGF$_{AB}$)-induced VEGF release in vascular smooth muscle cells by preventing activation of p38 MAPK and JNK (Oak et al, 2006). Olive oil polyphenols exert rapid inhibition of p38 and CREB phosphorylation leading to a downstream reduction in COX-2 expression in human colonic adenocarcinoma, Caco-2 cells (Corona et al, 2007).

Previously, we have already reported on significant anti-cancer activities of Quercetin, Siamois 1 and Siamois 2 polyphenols and the withasteroid Withaferin A,

which hold promise as dietary supplements in nutrition-based intervention in cancer treatment (Dechsupa et al, 2007; Kaileh et al, 2007). In this study we wanted to further investigate whether interference of Siamois polyphenols and withasteroids with NFκB-dependent apoptosis and inflammatory pathways can sensitize doxorubicin-resistant P-gp-overexpressing K562 erythroleukemic cells for capsase activation and late apoptosis.

Materials and Methods

Reagents and Chemicals

Quercetin, Kaempferol, and Eriodictyol were from Extrasynthèse (Genay, France) and 5,3'-dihydroxy-3,6,7,8,4'-pentamethoxyflavone (WP283) and the withasteroid Withaferin A was home-purified. These compounds were stored as 100 mM solution in DMSO at -20°C. Doxorubicin hydrochloride was kindly provided by by Dr. F. Offner (University Hospital UGent). Phorbol-12-myristate-13-acetate (PMA) was purchased from Sigma Chemical Company (St Louis, MO, USA) and stored as 1 mg/ml solution in DMSO at -20°C. Tumor Necrosis Factor (TNF) was Recombinant murine TNF, produced in *Escherichia coli* and purified in our laboratory to at least 99% homogeneity, had a specific biological activity of 8.58 x 10^7 IU/ml of protein as determined in a standard TNF cytolysis assay. Reference TNF (code 88/532) was obtained from the National Institute of Biological Standards and Control (Potters Bar, UK). Anti-IκBα, anti-p65 (C20), anti-p50 (NLS), anti-cRel (N), anti-RelB (C19), anti-Fra1(H50), anti-Nrf2 (C10) was from Santa Cruz Biotechnology (Santa Cruz, CA), anti-p38, anti-p44/42, , anti-cfos, anti-cjun, antijunB, anti-junD from Active Motif, anti-Sirt1 from Biomol, anti-Stat3 from Upstate, anti-histone-H3 antibodies from Abcam and anti-tubulin was from Sigma (Bornem, Belgium). The phospho-specific antibodies directed against p65 Ser536, p38 and p44/42 MAPK, cjun, Akt, MEK were from Cell signalling (Beverly, CA).

Cell culture and Cytotoxicity assay

Murine fibrosarcoma L929sA cells were maintained in Dulbecco's modified Eagle's medium supplemented with 5% newborn calf serum, 5% fetal calf serum, 100 units/ml penicillin, and 0.1 mg/ml streptomycin. Twenty-four hours before induction, cells were seeded in multiwell dishes such that they were confluent at the time of the experiment.

Doxorubicin-sensitive erythroleukemic cells (K562) and doxorubicin-resistant erythroleukemic cells (K562/adr) which overexpress P-gp were grown in RPMI1640 medium supplemented with 10 % fetal calf serum, 100 units/ml penicillin, and 0.1 mg/ml streptomycin, in an incubator at 37°C, 95% humidified, 5% CO_2. Cultures initiated at a density of 10^5cells/ml grew exponentially to about 10^6 cells/ml in 3 days. K562/adr cell line was cultured in RPMI 1640 medium in the presence of 100 nM doxorubicin for 72 h, after that the cells were grown in RPMI 1640 medium without doxorubicin for 2 weeks before the experiments. For the assays and in order to have cells in the exponential growth phase, cultures were initiated at 5 x 10^5 cells/ml and used 24 h later, reaching a density of about 8-10 x 10^5 cells/ml.

The cytotoxicity assay was performed as described previously (Reungpatthanaphong et al, 2003). Cells (5 x 10^4 cells/ml) were incubated in the presence of various concentrations of compounds tested. The viability of cells was determined by MTT reduction. The concentration of compound required for 50% inhibition of the proliferation of cells (IC50) was determined by plotting the percentage of cell growth inhibition (%IC) versus the compound concentration when measured at 72 h.

Apoptosis assay

Cells were washed with ice-cold phosphate-buffered saline (PBS) after treatment, and 5 x 10^5 cells were stained with annexin V (AnnV)-FITC during 15 min in the dark followed by propidium iodide (PI) staining (human AnnexinV–FITC Detection kit, Bender MedSystems Diagnostics, Vienna, Austria). The stained cells (10^4 cells) were measured by flow cytometry (Cytomics FC500 1 laser, Beckman Coulter, Fullerton, USA) and results were expressed as percentage living (AnnV–, PI–), early apoptotic (AnnV+, PI–), and late apoptotic/dead cells (AnnV+, PI+). The percent of living cells was normalized to 100% living cells incubated in control medium with 0.1% DMSO. All measurements were made in duplicate and averaged.

Measurement of caspase-3 activity

After appropriate induction, cells were washed with ice-cold PBS and the cytosolic cell lysate was prepared as described previously (Vanden Berghe et al, 2004). Measurement of caspase-3 activity was carried out by the incubation of cytosolic cell

lysate with fluorogenic substrates, Ac-DEVD-AMC. The release of fluorescent AMC was monitored for 1 h at 37°C at 2-min time intervals in a fluorescence microplate reader (Packard Instrument Co.) using a filter with an excitation wavelength of 360 nm and a filter with an emission wavelength of 460 nm (Denecker et al, 2001; Vanden Berghe et al, 2003). Data are expressed as the increase in fluorescence as a function of time (Δfluorescence/min) normalized with that of cells incubated in control medium with 0.1% DMSO.

Reporter Gene Analysis

The full-size IL6 promoter reporter gene constructed p1168hu.IL6P-Luc+ and the recombinant plasmids p(IL6κB)$_3$50hu.IL6P-luc+ were described previously (Vanden Berghe et al, 1999; Vanden Berghe et al, 1998). Stable transfection of L929sA cells was performed by the calcium phosphate precipitation procedure according to standard protocols (Vanden Berghe et al, 1998). Luciferase and galactosidase reporter assays were carried out according to the manufacturer's instructions (Promega) and have been described previously (Vanden Berghe et al, 1998). Normalization of luciferase activity was performed by measurement of β-galactosidase levels in a chemiluminescent reporter assay Galacto-Light kit (Tropix, Bedford, MA). Light emission was measured in a luminescence microplate reader (Packard Instrument Co.). Luciferase activity, expressed in arbitrary light units, was corrected for the protein concentration in the sample by normalization to the co-expressed β-galactosidase levels. β-Galactosidase protein levels were quantified with a chemiluminescent reporter assay Galacto-Light kit (Tropix).

Western blot analysis

For the western blot analysis of total cell lysate, cells were washed with ice-cold PBS before lysis in SDS sample buffer (62.5 mM Tris-HCL, 2% w/v SDS, 10% glycerol, 50mM DTT and 0.01% w/v bromophenol blue). To shear DNA and reduce sample viscosity, lysates were sonicated for 1 min in a water bath sonicater and then heated to 95°C for 5 min, after which they were immediately cooled on ice and microcentrifuged for 5 min. For the western blot analysis of nuclear extract, the nuclear proteins were suspended in SDS sample buffer at the same concentration. The protein samples were separated by 10% SDS-PAGE and electrotransferred onto a nitrocellulose membrane.

Blots were probed using the appropriate antibodies and the immunoreactive protein was detected using enhanced chemiluminescence reagents on a Kodak Imager system. Quantification of the chemiluminescence was done using the Quantity One software (BioRad, Nazareth Belgium).

Electrophoretic Mobility Shift Assay (EMSA)

After treatment, cells were washed with ice-cold PBS and pelleted in 1 ml PBS by centrifugation for 10 min at 2600 rpm (4°C). Preparation of nuclear extracts has been described previously (Vanden Berghe et al, 1999). For EMSA, equal amounts of protein were incubated for 25 min with an NFκB-specific ^{32}P-labeled oligonucleotide and binding mix as described previously (Kaileh et al, 2007). For supershift assay, antibodies were preincubated to the sample of interest for 10 minutes prior to incubation with radiolabelled probe (Plaisance et al, 1997). Labeling of the oligonucleotides was performed with [α-^{32}P]-dCTP by using Klenow enzyme (Boehringer Mannheim). For EMSA competition assays, 100 fold excess of unlabelled NFκB oligonucleotide was added to the binding mix. The NFκB oligonucleotide comprises the sequence: 5'-AGCTATGT**GGGTTTTCCC**ATGAGC-3', in which the single IL6 promoter-derived NFκB motif is bold and underlined. Samples were loaded on a 6% polyacrylamide gel run in 0.5 X TBE buffer (pH 8) and complexes formed were analysed using Phosphor Imager Technology.

RNA isolation and real-time Q-PCR analysis

Total RNA was extracted with the acid guanidinium thiocyanate-phenol-chloroform method using the Trizol reagent (Invitrogen, Merelbeke, Belgium). Reverse transcription was performed on 500 ng of total RNA in a 30 µl total volume. For normalization, cDNA concentrations in each sample were determined prior to quantitative real-time PCR (Q-RT-PCR). The Q-RT-PCR was performed on 5 µl of each condition using Invitrogen Sybr green platinum Supermix-UDG on a iCycler apparatus (Bio-Rad, Eke, Belgium). All amplifications were performed in duplicate or triplicate, and data were analysed using Genex software (Bio-Rad, Eke, Belgium). Data were expressed as mRNA expression normalized with that of cells incubated in control medium with 0.1% DMSO. QPCR primers are summarized in Table 1.

Table 1
Primers sequences used in the real-time Q-PCR. Note: the abbreviations used in the table: FW indicates forward; REV, reverse.

IL6 FW	GACAGCCACTCACCTCTTCA
IL6 REV	AGTGCCTCTTTGCTGCTTTC
IL8 FW	GCTCTCTTGGCAGCCTTCCTGA
IL8 REV	ACAATAATTTCTGTGTTGGCGC
A/BFL-1 FW	GATTTCATATTTTGTTGCGGAGTTC
A/BFL-1 REV	TTTCTGGTCAACAGTATTGCTTCAG
MCP1 FW	ACTCTCGCCTCCAGCATG
MCP1 REV	TTGATTGCATCTGGCTGAGC
A20 FW	CCTTGCTTTGAGTCAGGCTGT
A20 REV	TAAGGAGAAGCACGAAACATCGA
CYCLIND1 FW	CGCCCCACCCCTCCAG
CYCLIND1 REV	CCGCCCAGACCCTCAGACT
VEGF FW	GCCTCCCTCAGGGTTTCG
VEGF REV	GCGGCAGCGTGGTTTC
MDR1 FW	CTGCTTGATGGCAAAGAAATAAAG
MDR1 REV	GGCTGTTGTCTCCATAGGCAAT

Results

Siamois polyphenols and the withasteroid Withaferin A dose dependently inhibit NFκB-driven reporter gene expression

As anti-cancer properties of various polyphenols have been linked to inhibition of the inflammatory transcription factor NFκB, we first compared potential anti-inflammatory properties of the Siamois polyphenols quercetin, kaempferol, eriodictyol, WP283 and the withasteroid Withaferin A (Fig. 1A) in NFκB-driven reporter gene assays. First, we performed a dose reponse experiment in L929sA cells, stably transfected with a TNF-inducible NFκB-driven reporter gene construct with a minimal IL6 promoter ($p(IL6\kappa B)_3$-50hu.IL6P-luc+) and a constitutively expressed reporter gene construct (pPGκBGeobpA) controlled by the phosphoglycerokinase promoter (Vanden Berghe et al, 1999) for normalization of reporter gene expression. Upon TNF treatment, significant promoter induction can be observed with the NFκB-driven reporter gene construct, which can be reversed with quercetin, kaempferol, eriodictyol, WP283 or Withaferin A in a dose-dependent manner. IC50 values for NFκB inhibition for the different Siamois polyphenols vary in the concentration range of 30 to 50 μM and 0.5-1 μM for Withaferin A (Fig. 1B).

Siamois polyphenols and Withaferin A inhibit endogenous NFκB target gene expression in K562 and K562/Adr cells, irrespective of doxorubicin sensitivity

To validate our reporter gene expression results in more specific cancer settings, we further studied Siamois effects in K562 and K562/Adr cells, which may demonstrate different NFκB activation status related to doxorubicin sensitivity (Bednarski et al, 2008). Since NFκB hyperactivation is involved in chemoresistance, we next evaluated whether different types of NFκB inhibitors may have different effects on endogenous NFκB target genes in K562 and K562/Adr cells, involved in inflammation, metastasis (IL6, IL8, mcp1, A20), cell cycle (cyclinD1), angiogenesis (VEGF), multidrug resistance (mdr1/P-gp), and apoptosis (A1/Bfl1). Cells were pretreated with Siamois polyphenols or Withaferin A for 2 h, either or not following 3 h treatment of PMA, after which RNA was isolated and mRNA levels of interest were quantified by QPCR with specific primers. As illustrated in Fig. 2, NFκB target genes are potently induced by PMA in both cell types. Surprisingly, NFκB target genes are differentially expressed in K562 as compared to K562/Adr cells. More particularly, whereas IL6, IL8, MCP1, and A1/Bfl1 reveal stronger transcription in K562 cells, A20, cyclinD1, VEGF, and P-gp, are preferentially expressed in K562/Adr cells.are preferentially expressed in K562/Adr cells. Furthermore, repression of PMA inducible NFκB target genes can be observed in K562 and K562/Adr cells, irrespective of levels of Mdr1/P-gp expression. Interestingly, although NFκB inhibitors can completely reverse PMA inducible effects of P-gp in K562/Adr cells, basal transcription levels cannot be reversed to background levels observed in K562 cells.

Finally, efficacy of target gene repression seems also to be compound and target gene specific. Altogether, these results demonstrate selective inhibitory effects of Siamois polyphenols and withasteroids on target genes involved in inflammation, metastasis, cell cycle, angiogenesis, multidrug resistance, and anti-apoptosis in doxorubicin sensitive and resistant K562 cells.

Siamois polyphenols and Withaferin A all prevent IκB degradation but selectively interfere with p38, ERK MAPK, MEK1 and Akt kinase activation

As NFκB target gene expression encompasses multiple regulatory steps, including IκB degradation, NFκB translocation, NFκB/DNA binding and NFκB transactivation. We next aimed to dissect which regulatory steps are affected by Siamois polyphenols in

K562 and K562/Adr cells. Since IκBα degradation is required for liberation and subsequent translocation of NFκB to the nucleus, we determined Siamois effects on PMA induced IκBα protein degradation in K562 and K562/Adr cells. As maximal degradation of IκBα is observed between 15-30 minutes after PMA treatment, we measure effects of Siamois polyphenols and Withaferin A on IκB degradation following 2 h pretreatment and 30 minutes cotreatment with PMA. From Fig. 3A, it can be observed that in both cell types 30 minutes of PMA treatment is able to reduce IκB levels. Furthermore, all tested compounds reduce IκB degradation in both cell types (Fig. 3B). Along the same line, all tested compounds significantly reduce basal and/or PMA inducible p65 Ser536 phosphorylation in both cell types. Altogether, these results suggest that activation of NFκB and subsequent translocation of NFκB for gene induction is significantly reduced in presence of Siamois polyphenols and the withasteroid Withaferin A.

As target gene-specific effects are also depending on p65 phosphorylation status and epigenetic settings, dynamically controlled by multiple kinase pathways , i.e. Akt, MAPK, MSK, PKA we next measured P-Akt, P-p38, P-ERK levels in the various experimental conditions in both cell types. A significant reduction of basal and PMA-induced P-Akt and P-p38 levels can be observed upon treatment with quercetin and kaempferol but not with Withaferin A in both K562 cell types (Fig. 3B), whereas P-ERK levels do not reveal significant inhibition (Fig. 3C). In contrast weak ERK stimulation could rather be observed with Withaferin A and quercetin (Fig. 3C). Western analysis against p38 and ERK protein levels confirms equal protein loading in the various experimental setups (Fig. 3C). Interestingly, Siamois polyphenols and Withaferin A demonstrate increased MEK1-phosphorylation in K562/Adr cells, suggesting that uptake of compounds is not impaired in P-gp overexpressing K562/Adr cells.

Altogether, besides significant inhibition of IκB degradation and NFκB p65 Ser536 activation by Siamois polyphenols and Withaferin A, compound-specific regulation of p38, ERK, Akt and MEK kinases could be observed, which may further interfere with nuclear transcriptional regulation of NFκB target genes (Cohen, 2009; Natoli et al, 2005; Viatour et al, 2005).

K562 and K562/Adr cells reveal distinct nuclear regulation of NFκB, AP1, Nrf2 and Sirt1 proteins

As K562 and K562/Adr demonstrate differential regulation of NFκB target genes, we next explored whether both cell types may show different nuclear regulation of potential cooperative transcription factors (i.e. AP1, Nrf2) or cofactors (Sirt1) which might coregulate NFκB target genes. As can be observed from Fig. 4, basal levels of nuclear NFκB p65, AP1 c-Jun, JunD and Fra1 are significantly increased but not of cRel and RelB. This confirms previous observations on doxorubicin resistant-MCF7 cells, in which AP1 transcription factors were demonstrated to be responsible for upregulation of P-gp/Mdr1 (Daschner et al, 1999). Furthermore, PMA treatment significantly increases nuclear levels of NFκB p65, RelB, c-Rel. Of special note, increased nuclear levels of Nrf2 upon PMA treatment are more pronounced in K562/Adr than K562 cells. Only recently, involvement of Nrf2 has been demonstrated in chemoresistance (Wang et al, 2008). Also in line with previous studies on the role of Sirt1 in chemoresistance, basal Sirt1 levels are slightly increased in doxorubicin-resistant K562/Adr cells. More particularly, Sirt1 was found to positively contribute in P-gp/Mdr1 expression (Chu et al, 2005). Altogether, our results demonstrate that activities of NFκB p65, AP1 cjun, junD, Fra1, Nrf2 transcription factors and Sirt1 cofactors are increased in doxorubicin resistant K562/Adr cells.

NFκB, AP1 DNA binding profiles in K562 and K562/Adr cells show qualitative and quantitative differences

To compare DNA-binding properties of NFκB, AP1 in K562 and K562/Adr cells, we performed electrophoretic gel shift mobility assays (EMSA) and supershift analysis in response to PMA stimulation. Fig. 5A reveals that both cell types show inducible NFκB/DNA binding, whereas basal NFκB/DNA binding is slightly elevated in doxorubicin-resistent K562/Adr cells, in line with observations that doxorubicin can elevate basal NFκB activation via DNA damage pathways (Gangadharan et al, 2009a). Interestingly, supershift analysis reveals subtle differences in the heterodimer/homodimer composition of DNA-bound NFκB and AP1 binding complexes in both cell types. Supershift analysis reveals at least three different NFκB/DNA binding complexes including p65-p65, p50-p65, and p50-p50. In K562/Adr cells, basal NFκB/DNA binding of the p50-p65 complex appearsrelative to K562 cells. Similarly, increased basal and inducible AP1 binding is detected in K562/Adr cells in comparison with K562 cells, in

line with increased levels of nuclear AP1 members. Furthermore, although both cell types demonstrate PMA inducible NFκB/DNA binding, K562 cells show higher intensity of p65-p65 heterodimers but comparable amounts of p50-p65 and p50-p50 DNA-binding complexes in comparison to K562/Adr cells (Fig. 5A). Concerning AP1-binding complexes, increased Fra1 levels can be detected in K562/Adr cells as compared to K562 cells. EMSA competition with excess unlabelled NFκB or AP1 DNA-binding motifs further demonstrates specificity of the DNA-bound NFκB, RBP-Jκ and AP1-binding complexes.

Siamois polyphenol quercetin, eriodictyol and Withaferin A strongly inhibit DNA binding of NFκB, AP1 and Nrf2

To verify whether transcriptional repression of target genes involved in inflammation, anti-apoptosis, angiogenesis, metastasis, drug resistance by Siamois polyphenols and Withaferin A may be the consequence of inhibition of NFκB, AP1 or Nrf2 TF/DNA binding in K562 and K562/Adr cells, we performed EMSA experiments with nuclear extracts from cells treated with PMA alone, or following pretreatment with Siamois polyphenols. As shown in Fig. 5B, basal constitutive p50-p50 and p50-p65 NFκB/DNA-binding activity in K562/Adr is increased as compared to K562 cells. PMA stimulation again increases p50-p50 and p50-p65 NFκB/DNA binding in both cell types whereas p65-p65 homodimers demonstrate stronger DNA binding in K562 only. Furthermore, treatment with different Siamois polyphenols and Withaferin A causes strong to moderate inhibition of the basal and inducible NFκB-and AP1-DNA binding complexes, as *shown in fig. 5B* (Plaisance et al, 1997). Along the same line, Nrf2/DNA binding is increased in K562/Adr cells as compared to K562 cells, whereas Siamois polyphenols and Withaferin A are able to reduce basal and PMA inducible Nrf2 binding in both cell types (Huang et al, 2000; Wang et al, 2008). Of the different Siamois polyphenols tested, quercetin and eriodictyol show the strongest inhibition of TF/DNA binding, whereas Kaempferol and WP283 are less effective. Nevertheless, transcriptional inhibition of the various target genes (Fig. 5B) by Siamois polyphenols and Withaferin A is regulated at multiple levels and depends on DNA-binding properties of NFκB, AP1, Nrf2 transcription factors, nuclear cofactor dynamics, as well as epigenetic settings (Dong et al, 2008; Natoli et al, 2005; Nowak et al, 2008; Vanden Berghe et al, 2006b). Of

special note, although Siamois polyphenols and Withaferin A are able to reverse inducible NFκB/DNA-binding in K562/Adr cells, basal NFκB/DNA-binding levels cannot be further decreased to levels observed in K562 cells.

Siamois polyphenols and Withaferin A reduce cell viability in both K562 and K562/Adr cells

K562 and K562/Adr cells which are sensitive or resistant to doxorubicin respectively, were incubated with doxorubicin, Withaferin A or Siamois polyphenols, including quercetin, kaempferol, eriodictyol, WP283 to evaluate cytostatic and/or cytotoxic activity of the various compounds. After 72 h, cell survival was determined by the MTT cell viability assay and the IC50 values are summarized in Fig. 6. Among Siamois polyphenols, WP283 and eriodictyol exhibit the strongest and weakest effects in mitochondrial reduction of tetrazolium salts to formazan. Of particular interest, K562 and K562/Adr cells reveal comparable sensitivity to Siamois polyphenols and Withaferin A, wheres IC50 values for doxorubicin show a 20-fold higher sensitivity in sensitive K562 cells, as compared to resistant K562/Adr cells. These results indicate a pronounced cellular resistance for doxorubicin as compared to Siamois polyphenols and Withaferin A.

Withaferin A, but not Siamois polyphenols, induces execution of apoptosis

Next, K562 and K562/Adr cells were incubated for 48 h with Siamois polyphenols or Withaferin A, followed by annexin V-FITC/PI double staining and FACS analysis to quantify early annexin V-FITC positive) and late (annexin V-FITC/PI double positive) apoptotic cells. The relative percentage apoptotic/living cells in the different experimental setups in K562 and K562/Adr cells, following 48h treatment are represented as a bar graph in Fig. 7. Interestingly, although both cell types show comparable early apoptotic cell populations in presence of the different Siamois polyphenols, late apoptotic cells only accumulate in K562 cells. In contrast to Siamois polyphenols, only Withaferin A is able to trigger late apoptosis inK562/Adr cells. Furthermore, although the concentrations applied of the different Siamois polyphenols closely relate to the IC50 values determined in MTT assay (Fig. 6), FACS analysis (Fig. 7) reveals significiant variation in apoptosis efficacy between the different polyphenol compounds. The latter suggests significant discrepancies between MTT cell viability assays revealed by

mitochondrial reduction of tetrazolium salts and cell survival score measured by Annexin V/PI apoptosis FACS assay. Indeed, it is of utmost importance to perform multiple, methodologically unrelated assays to quantify dying and dead cells (Galluzzi et al, 2009). Next, as apoptotic threshold in compound-treated K562/Adr cells may be higher due to elevated basal anti-apoptotic activity of NFκB, AP1 and Nrf2, we wanted to further evaluate whether increasing activity of NFκB, AP1 and Nrf2 by PMA treatment in K562 cells could similarly protect compound treated K562 cells from late apoptosis in analogy to K562/Adr cells. However, although the relative number of late apoptotic cells decreases upon cotreatment of K562 cells with PMA and Siamois inhibitors, execution of apoptosis is not completely blocked bacause Siamois polyphenols are able to partially counteract PMA effects on NFκB, AP1 and Nrf2. Along the same line, Siamois polyphenols cannot overcome late apoptosis block in K562/Adr cells, despite efficient inhibition of NFκB, AP1 and Nrf2. This suggests that execution of apoptosis in K562/Adr cells is only in part determined by transcriptional activity of NFκB, AP1 and Nrf2. Remarkably, Withaferin A which inhibits NFκB, AP1 and Nrf2 to a similar extent as quercetin in K562/Adr cells is able to trigger late apoptosis and overcome apoptosis block in K562/Adr cells indicating that Withaferin A may also affect other death-inducing pathways/mechanisms.

Withaferin A, but not Siamois polyphenols, induce caspase-3 activation in K562/Adr cells

In addition to propidium iodide as a late apoptotic FACS marker, we next measured biochemical activation of the executioner caspase-3 in K562 and K562/Adr cells exposed to PMA, Siamois polyphenols and/or Withaferin A. Time kinetic analysis (2-72 h treatment) of caspase-3 activation of K562 cells exposed to quercetin revealed maximal caspase-3 activity at 12 h (data not shown) in a fluorescent caspase substrate assay. In this respect, K562 and K562/Adr cells were treated again for 12 h with PMA??, Siamois polyphenols and/or Withaferin A, after which caspase3 activity present in the cell lysates was again measured. From Fig. 8 it can be noticed that Siamois polyphenols only increase caspase-3 activation in K562 cells but not in K562/Adr cells, in line with lack of late apoptosis observed in K562/Adr cells. In contrast to Siamois polyphenols, Withaferin A is able to trigger caspase-3 activation in both cell types. This demonstrates

that Withaferin A in addition to transcriptional inhibition of NFκB, AP1 and Nrf2, may restore apoptosis and caspase-3 activation in P-gp overexpressing K562/Adr cells which can chemosensitize K562/Adr cells for cancer drug treatment.

Discussion and Conclusion

Extensive studies indicate that both hyperactivated NFκB and overexpression of multidrug transporters play important roles in cancer chemoresistance (Bednarski et al, 2008; Bentires-Alj et al, 2003; Chu et al, 2005; Gottesman et al, 2002; Rayet & Gelinas, 1999; Thevenod et al, 2000). Since expression of the multidrug transporter P-gp was found to be NFκB-dependent, it is believed that NFκB-inhibitors can decrease P-gp expression and restore chemosensitivity (Nakanishi & Toi, 2005; Surh, 2003). However, what emerges from our studies is a more complex picture. Previously, we have already demonstrated apoptosis of MDA-MB435 cells in presence of Siamois polyphenols in a xenograft model in vivo (Dechsupa et al, 2007). Furthermore, the NFκB inhibitor Withaferin A has been described as a promising drug for cancer chemotherapy and radiosensitization (Devi, 1996; Devi et al, 1996; Kaileh et al, 2007). Here we further analysed whether Withaferin A or Siamois polyphenols quercetin, kaempferol, eriodictyiol, and WP283 hold therapeutic promise as NFκB inhibitors for chemosensitization of doxorubicin resistant K562/Adr erythroleukemia cells. In NFκB reporter gene studies, we measured dose dependent repression of IL6with Siamois polyphenols quercetin, kaempferol, eriodictyiol, and WP283 with IC50 values in the range of 0,1-50μM respectively. Furthermore, upon comparing endogenous gene expression of NFκB target genes, we observe similar potencies in NFκB-dependent gene repression by Siamois polyphenols in K562 and K562/Adr cell types, although both cell types reveal differential expression of specific NFκB target genes. More particularly, K562 cells reveal a predominant inflammatory gene expression profile (i.e. strong expression of IL6, IL8, MCP1, and A1/Bfl1), whereas K562/Adr cells demonstrate a more tumorigenic pattern (i.e. strong expression of A20, cyclinD1, VEGF, and mdr1A1/Bfl1). As such, we further studied NFκB signaling mechanisms and coregulatory pathways which may be responsible for differential NFκB target gene expression/inhibition and apoptosis sensitivity for Withaferin A and Siamois polyphenols. Upon characterization of the major NFκB activation and transactivation pathways, we

found differential regulation of NFκB activity by Withaferin A and quercetin, kaempferol, eriodictyol and WP283. Interestingly, IκB degradation and NFκB/DNA binding was significantly reduced by all compounds tested in both cell types, among whichWithaferin A, quercetin and eriodictyol showing the most potent inhibition, and kaempferol and WP283 much weaker and variable inhibition. Remarkably, increased levels of basal NFκB binding in K562/Adr cells cannot be inhibited by Siamois polyphenols in contrast to inhibition of inducible NFκB/DNA-binding. Furthermore, relative composition of NFκB/DNA binding complexes reveals that K562 cells contain much higher levels of p65-p65 homodimers. Of particular interest, the inflammatory cytokine IL8 was found to preferentially bind p65-p65 homodimers instead of p50-p50 and p50-p65 dimers (Kunsch 1993), which could explain strong expression of inflammatory cytokines in K562 cells. From another perspective, NFκB dimer composition may also depend on the repertoire posttranslational modifications present on NFκB (Benayoun & Veitia, 2009; Jacque et al, 2005; Viatour et al, 2005). More specifically, we found variable compound-specific effects on p38 MAPK, MEK1, Akt kinase pathways, which may also interfere with NFκB transcription factor composition and/or activity.

Finally, besides phosphoregulation of transcription factors, acetylation by cofactors (CBP, HDAC, Sirtuin) has recently added an additional control of NFκB transcription factor activity (Calao et al, 2008; Natoli, 2009; Zhong et al, 2002). Of special note, as doxorubicin was found to increase Sirt1 HDAC levels (Chu et al, 2005), we compared nuclear Sirt1 levels in both cell types and observed a significant increase in Sirt1 protein in K562/Adr. As such, we cannot exclude that in addition to kinases also Sirt HDACs may contribute in cell-specific phosphoacetylation control of TF/DNA binding and transcriptional activity and may prevent NFκB p65 homodimer formation. In addition to cell specific regulation of NFκB, it can be observed from Fig. 4 that also AP1 members (i.e. Fra1, cjun and junD) and Nrf2 are differentially expressed in both cell types. As such, we can also neither excludecompound-specific kinase effects on these transcription factor families, since various NFκB target genes involved in inflammation, metastasis, angiogenesis and drug resistance are also coregulated by AP1 and Nrf2 (Daschner et al, 1999; Eferl & Wagner, 2003; Wang et al, 2008)

Most surprisingly, although inhibition of NFκB activity in general contributes in chemosensitisation of cancer cells (Bednarski et al, 2008; Gangadharan et al, 2009b), treatment of K562/Adr cells with Siamois polyphenols failed to cleave caspase-3 and trigger late apoptosis to similar levels as observed in K562 cells, although efficacy of NFκB inhibition and initiation of early apoptosis by Siamois polyphenols is similar in doxorubicin-sensitive and resistant cell types. This is in line with previous reports on drug resistance, which describe that P-glycoprotein inhibits cytochrome c release and caspase-3/8 activation, but not formation of the death-inducing signal complex (Friedrich et al, 2001; Ruefli et al, 2002; Smyth et al, 1998). The fact that Siamois polyphenols are able to completely ablate NFκB target gene expression, hyperactivate MEK1 and trigger early apoptosis in K562/Adr cells argues against the hypothesis that Siamois polyphenols may not be uptaken or are secreted out of the cell because of hyperactivated P-gp activity in K562/Adr cells. As such, P-gp overexpression confers resistance to a wide range of caspase-dependent apoptotic agents not only by removing drugs from the cell, but also by inhibiting the activation of proteases involved in apoptotic signaling (Johnstone et al, 1999). Only a few drugs are reported to overcome this P-gp/Mdr phenotype and most of them are molecules that induce cell death in a caspase-independent manner (Ruefli et al, 2000). Interestingly, in analogy to some specific glutathione S transferase inhibitors (NBDHEX) and mitochondria-targeting drugs (oligomycin) (Ascione et al, 2009; Li et al, 2004; Turella et al, 2006), Withaferin A was found to bypass the P-gp resistance and trigger late apoptosis in a caspase-3-dependent manner in K562/Adr cells., demonstrating that the block of caspase-dependent apoptosis by P-gp is reversible. Further investigation is needed to explore how exactly Withaferin A is able to counteract P-gp activities. Of particular interest, it can not be excluded that thiolreactivity of Withaferin A interferes with cysteine-sensitive P-gp protein folding steps and/or P-gp protein function (Loo & Clarke, 1999; Loo & Clarke, 2001).

In conclusion, we found that transcriptional inhibition of NFκB-, AP1- and Nrf-driven target genes involved in inflammation, metastasis, angiogenesis, drug resistance is not sufficient to overcome the P-gp-coupled block of caspase-dependent apoptosis in K562/Adr cells. In contrast, the withasteroid Withaferin A was found to restore caspase-3 activation and trigger apoptosis in K562/Adr cells which makes this an attractive compound to circumvent drug resistance and to elicit cell death in chemoresistant cell

types. However, Siamois polyphenols may have therapeutical benefit upon suppression of cancer-promoting inflammatory cytokines and factors involved in cancer progression, although less effective in eradication of tumor cells by triggering apoptosis. The latter strategy may be beneficial to globally retard progression of aggressive refractory tumors, instead of chemotherapy of refractory tumors, which may further select for clonal expansion and evasion of chemoresistant and/or metastatic cancer cells.

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Figure legends

Fig. 1. Siamois polyphenols and withasteroids dose-dependently inhibit NFκB-dependent reporter gene expression. A) Chemical formulas of quercetin, kaempferol, eriodictyol, WP283, and Withaferin A. **B)** L929sA cells, stably transfected with p(IL6κB)$_3$50hu.IL6P-luc+, were left untreated or were pretreated with a concentration range of 100-50-25-12,5 μM Siamois polyphenol (quercetin, kaempferol, eriodictyol, WP283) or 3-1.5-0,75-0,38 μM Withaferin A for 2 h, and then stimulated with 2000 IU/ml TNF for 4 h. NFκB-dependent reporter gene expression was normalized for PGK housekeeping reporter gene expression.

Fig. 2. Siamois polyphenols and withasteroids inhibit endogenous NFκB-dependent transcription in K562 and K562/Adr cells. K562 and K562/Adr cells were pretreated with 100 μM of quercetin, kaempferol, eriodictyol, WP283, or 6 μM of Withaferin A for 2 h followed by incubation with PMA (0,1μg/ml) for 3 h. Total RNA was isolated and mRNA was converted into cDNA. Relative mRNA levels were quantified by QPCR by specific primersets for **A)** IL6, IL8, A1/Bfl1, MCP1, **B)** A20, cyclinD1, VEGF, mdr1. Specific mRNA transcription levels were normalized by transcription levels of cells incubated in control medium with 0.1% DMSO.

Fig. 3. Selective effects of Siamois polyphenols and withasteroids on the NFκB signaling pathway. K562 and K562/Adr cells were either or not pretreated with 100 μM of quercetin, kaempferol, eriodictyol, WP283 or 6 μM of Withaferin A for 2 h followed by incubation with PMA (0,1μg/ml) for 30 minutes. Total cell lysates were prepared in SDS-Laemmli sample buffer and extracts were analysed for protein expression levels of **A-B)** IκB, p65, P-Ser536 p65, P-p38, P-Akt, tubulin, **C)** P-MEK1, P-ERK, ERK, respectively.

Fig. 4. K562 and K562/Adr cells reveal different nuclear regulation of NFκB, AP1, Nrf2 transcription factors and Sirt1 cofactors. K562 and K562/Adr cells were treated with PMA (0,1μg/ml) for 30 minutes. Nuclear cell lysates were prepared in SDS-Laemmli sample buffer and extracts were analysed for protein expression levels by Western analysis of NFκB p65, RelB, cRel, Nrf2, AP1 cjun, junD, Fra1, Sirt1, respectively. Comparable protein loading was verified with Histone H3 antibodies.

Fig. 5. K562 and K562/Adr cells show qualitative and quantitative differences in NFκB and AP1 DNA binding profiles. A) K562 and K562/Adr cells were pretreated with PMA (0,1μg/ml) for 30 minutes. Nuclear lysates were analysed for NFκB/DNA and AP1/DNA binding with a radiolabelled IL6 κB site- or AP1-motif containing probe. Binding complexes formed were analysed by EMSA. Loading of equal amounts of protein was verified by comparison with the binding activity of the repressor molecule recombination signal sequence-binding protein Jκ (RBP-Jκ). Specificity of the various complexes bound is demonstrated by supershift analysis with NFκB and AP1 specific antibodies, as well as by competition with 100-fold excess cold oligo. **B)** K562 and K562/Adr cells were pretreated with 100 μM of quercetin, kaempferol, eriodictyol, WP283, or 6 μM of Withaferin A for 2 h followed by incubation with PMA (0,1μg/ml) for 30 minutes. Cell lysates were fractionated for cytoplasmic and nuclear extracts which wereanalyzed for NFκB, AP1, or Nrf2-dependent DNA binding with specific radiolabelled probes. Binding complexes formed were analysed by EMSA.

Fig. 6. Mitochondrial effects of Siamois polyphenols and withasteroids in K562 and K562/Adr cells. K562 and K562/Adr cells were treated with various concentrations quercetin, kaempferol, eriodictyol, WP283 or Withaferin A for 72 h. Cell survival was determined by mitochondrial MTT assay and IC50 values were determined for cytotoxicity of the different compounds.

Fig. 7. Apoptotic effects of Siamois polyphenols and withasteroids in K562 and K562/Adr cells. Determination of early (AnnexV+, PI-) and late (AnnexV+, PI+) apoptotic cells by flow cytometric evaluation (AnnexinV-FITC and PI staining) of mean normalized percentages of living K562 and K562/Adr cells, either left untreated or treated with 100 μM of quercetin, kaempferol, eriodictyol, WP283 or 6 μM of Withaferin A, or cotreated with PMA (0,1μg/ml) for 48 h.

Fig. 8. Caspase 3 activation by Siamois polyphenols and withasteroids in K562 and K562/Adr cells. K562 and K562/Adr cells were either left untreated or treated with PMA (0,1μg/ml) and 100 μM of quercetin, kaempferol, eriodictyol, WP283 or 6 μM of Withaferin A for 12 h. Caspase-3 activity was determined by a fluorescent *in vitro* assay upon incubation of cell lysate with Ac-DEVD-fmk substrate.

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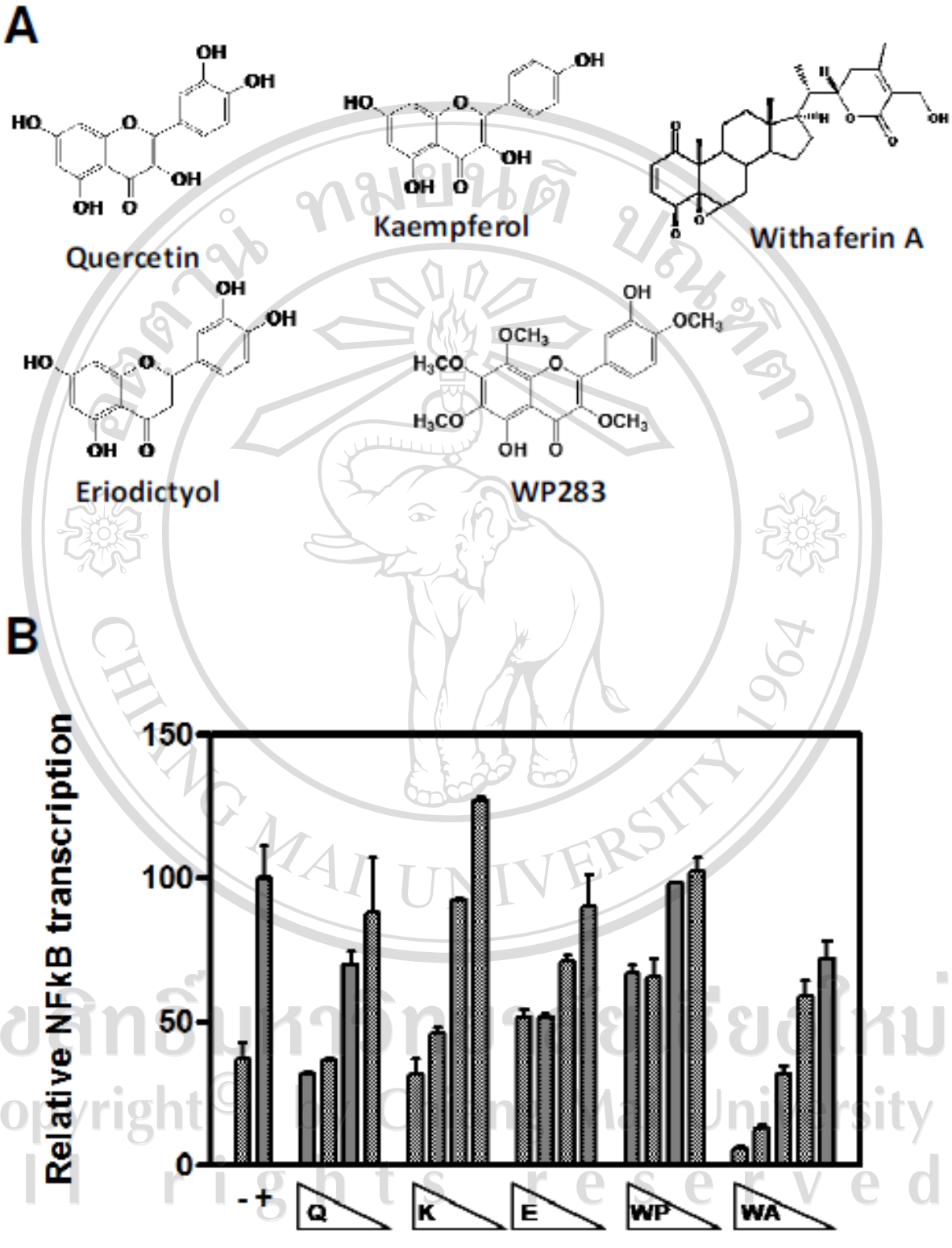
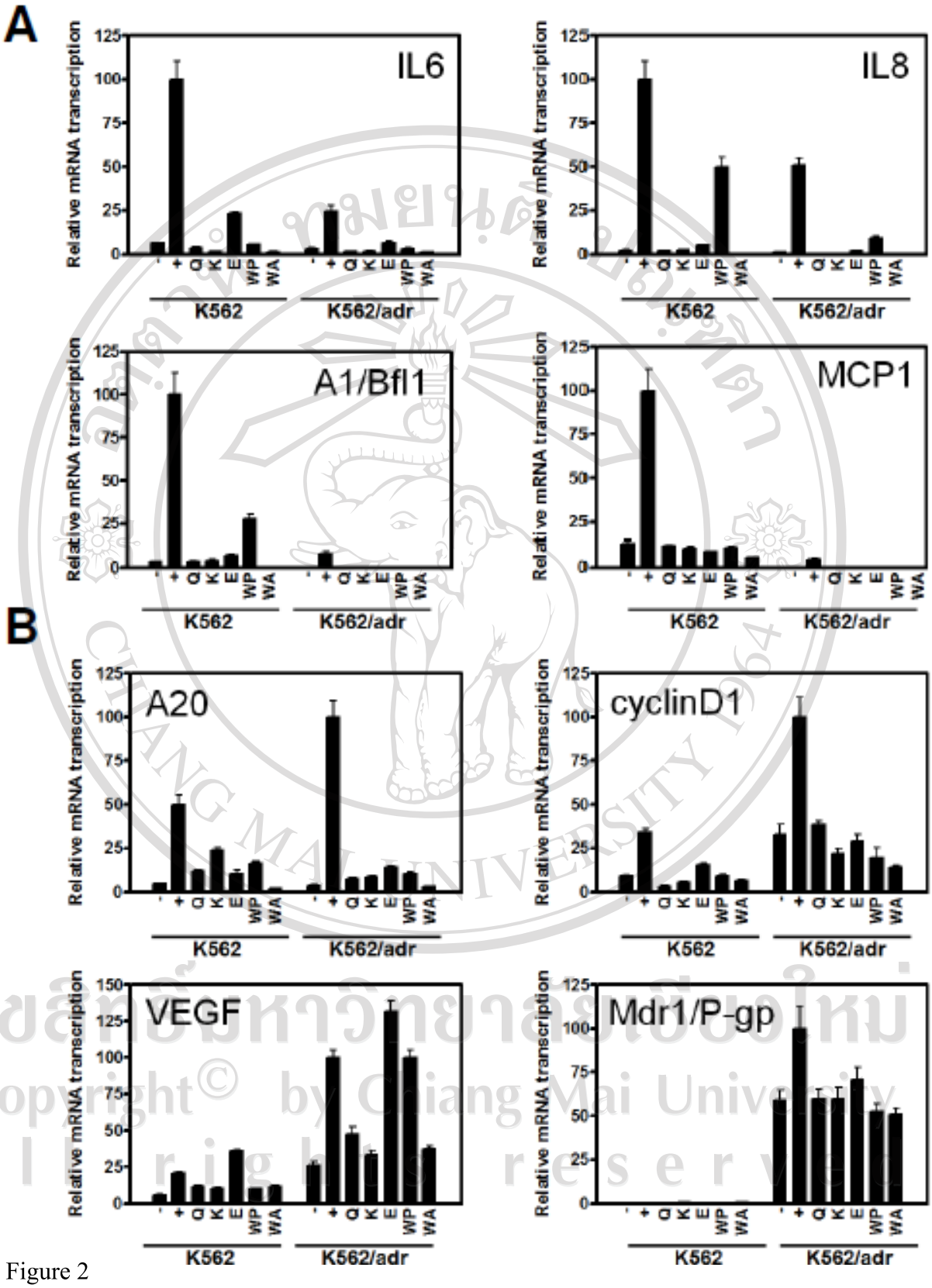


Figure 1



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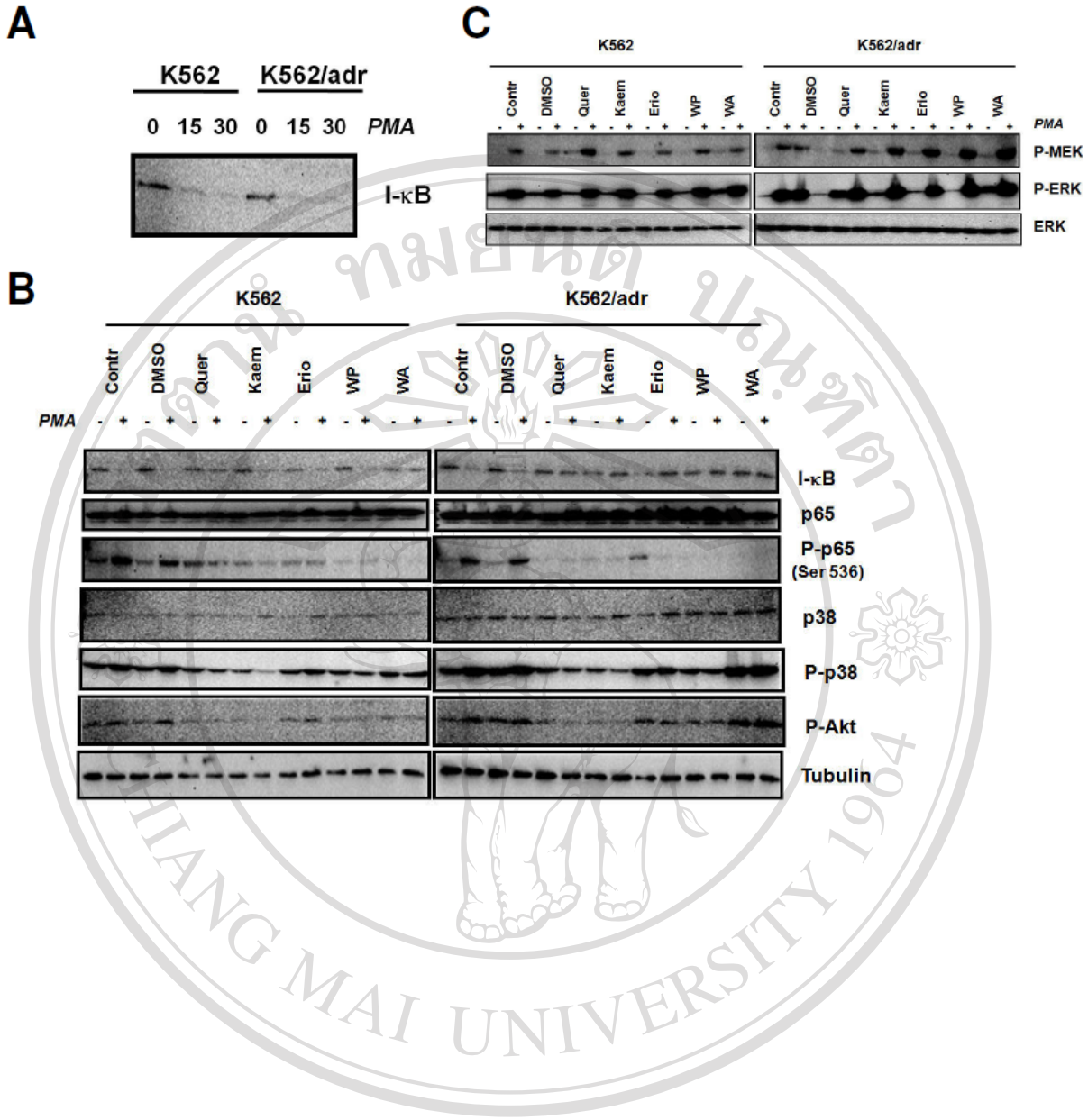

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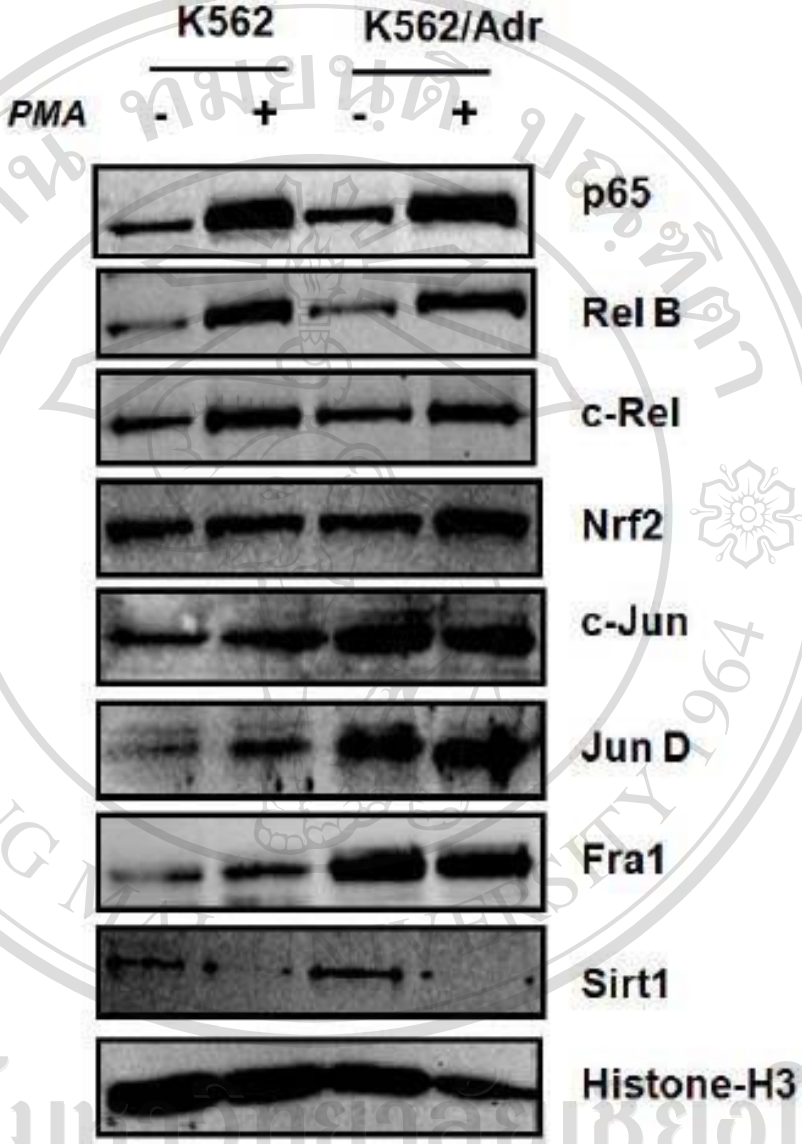

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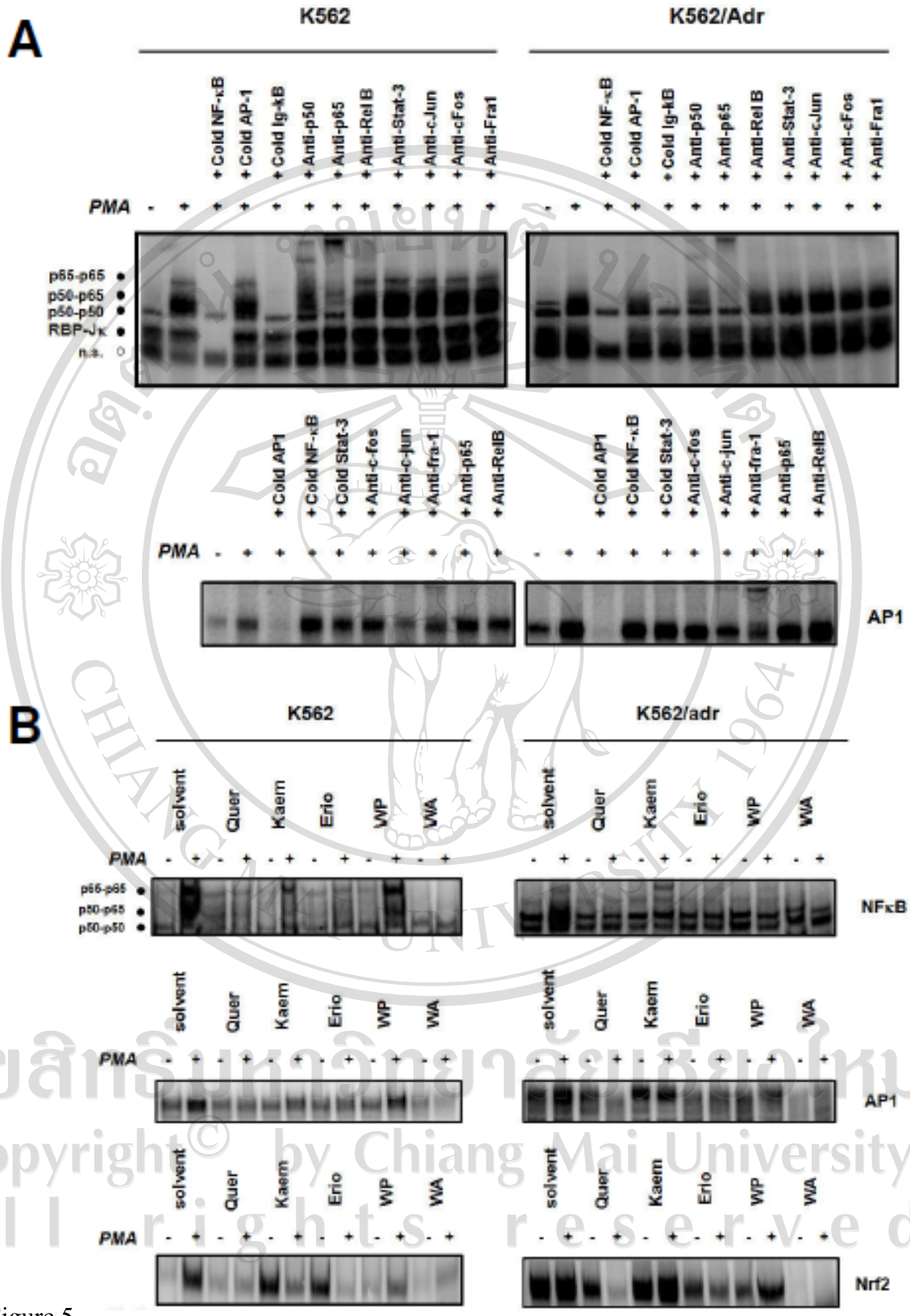

Figure 5

IC50 value of interested compounds in K562 and K562/adr cells

Compounds	IC50 (μM)	
	K562	K562/adr
Quercetin	26 ± 2	32 ± 3
Kampferol	39 ± 3	60 ± 4
Eriodictyol	> 100	> 100
WP283	0.03 ± 0.02	0.03 ± 0.01
Withaferin A	0.2 ± 0.02	0.2 ± 0.06
Doxorubicin	0.2 ± 0.04	4.1 ± 1.0

Figure 6

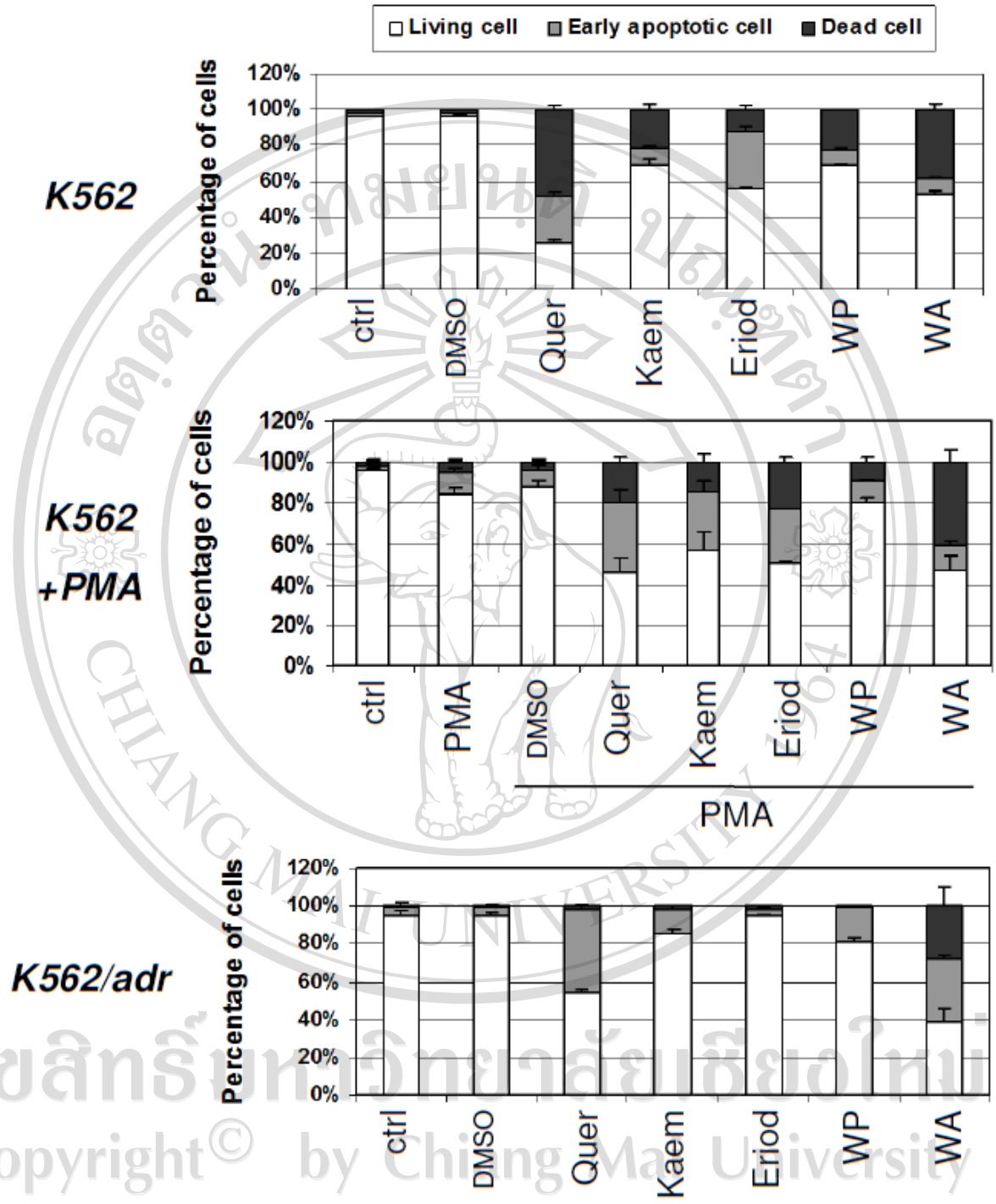

Figure 7

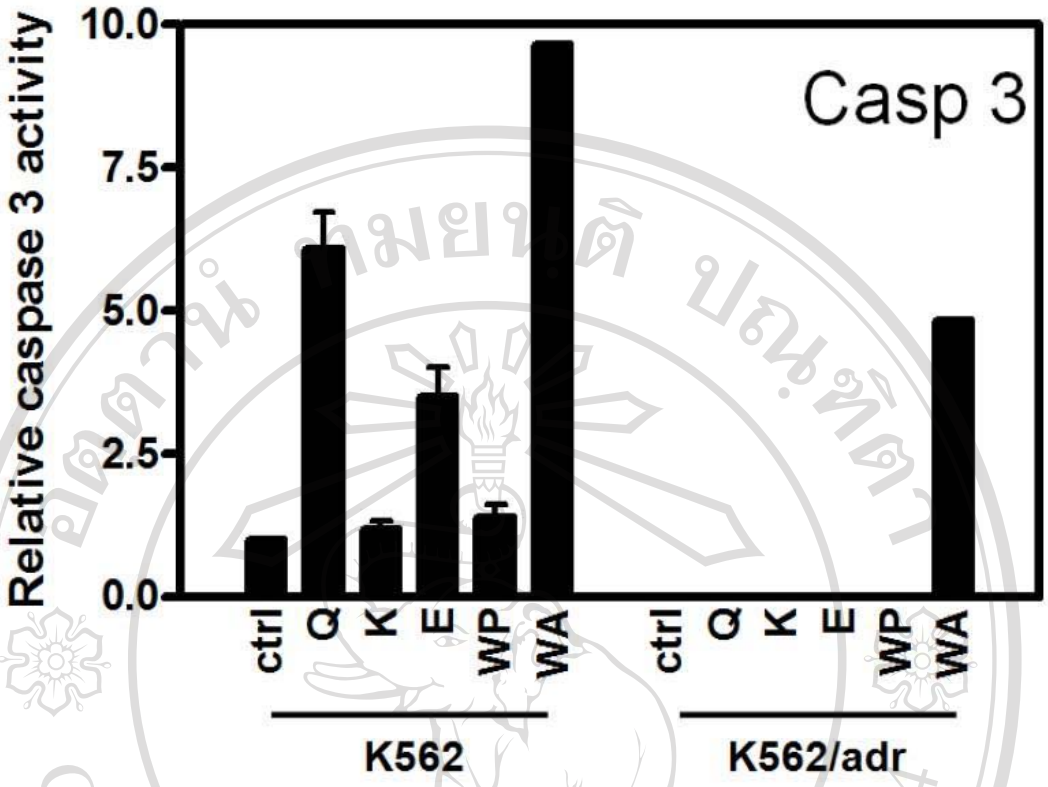

Figure 8

APPENDIX B

Research article

The intracellular targets and apoptosis-inducing activity of Siamois® in drug-sensitive and drug-resistant cancer cells

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Abstract

Siamois® is obtained from homemade Siamois red wine and is a rich source of flavonoids that have previously been reported to have potential anticancer affects both *in vitro* and *in vivo* (Dechsupa *et al*. in Cancer biology & Therapy, 2007). In this study, the intracellular targets of Siamois® in living normal myoblasts and drug-sensitive and –resistant human cancer cells were visualized using acridine orange, rhodamine B, and pirarubicin with an inverted fluorescence microscope. The cellular energetic state (ATP, ADP and AMP levels) and spontaneous changes in the mitochondrial membrane potential of treated cells were also examined. Siamois1 and Siamois2 significantly inhibited cell growth of both drug-sensitive and multidrug resistant cancer cells. Siamois 1 exhibited about 30 to 100 fold greater anticancer activity than Siamois 2; the IC_{50} values were 0.05 ± 0.01 $mg.mL^{-1}$ for MDA-MB 435, 0.12 ± 0.02 $mg.mL^{-1}$ for K562 and K562/adr, and 0.18 ± 0.02 $mg.mL^{-1}$ for GLC4 and 0.10 ± 0.2 $mg.mL^{-1}$ GLC4/adr cells. Siamois® polyphenols affected the mitochondrial function of both normal myoblasts and cancer cells, but only induced dramatic mitochondrial depolarization, a decrease in ATP content, and apoptosis in cancer cells. Siamois 1 and Siamois 2 exhibited similar cell death efficacy in all cancer cell lines tested, with approximately 50% apoptosis induction when cells were exposed to150 $\mu g.mL^{-1}$ for 24 h. Thus, Siamois 1 and Siamois 2 effectively limit cancer cell growth through apoptototic mechanisms.

Key words: Red wine polyphenols, human myocytes, doxorubicin, multidrug resistance phenomenon, apoptosis-inducing activities, Siamois®, mitochondria

Introduction

Red wine is a rich source of biologically active phytochemicals known as polyphenols, which are beneficial against degenerative conditions such as cardiovascular disease and carcinogenesis.[1,2] The ability of red wine polyphenols to prevent cancer has been the subject of several recent studies.[3-5] Dolara P *et al.* reported that polyphenols from red wine inhibit the process of chemical colon carcinogenesis in rodents, modify colon microbial ecology, reduce colonic mucosa DNA oxidation, and have complicated effects on gene regulation that may affect the mucosal response to inflammatory and carcinogenic agents.[4] It is not clear at present how the observed variations in gene regulation are specifically connected to protection from oxidative damage and/or inhibition of carcinogenesis.

Polyphenols are considered to be effective general inhibitors of cancer cell growth, and induce apoptosis of various cancer cell lines, including human colon carcinoma[6] and prostate cancer cells.[7,8] The relationship between the chemical structure of polyphenols and their anticancer effects has been widely studied *in vitro* and *in vivo*, and polyphenol concentrations required to restrict cancer cells vary depending on the cancer cell type.[9] Polyphenols have been shown to affect the function of multidrug-resistance transporters such as P-glycoprotein and MRP proteins.[10, 11] Multidrug resistance is the major cause of failure in cancer chemotherapy. We recently reported that flavonoids abundantly found in red wine, such as quercetin, apigenin, kaempferol and eriodictyol, significantly inhibited the growth of several cancer cell lines with similar efficacy ($IC_{50} \approx 15 \pm 2$ μM against human erythromyelogenous leukemic drug-sensitive K562 and drug-resistant, P-glycoprotein overexpressing K562/*adr* cells and human small cell lung carcinoma drug-sensitive GLC4 and drug-resistant, MRP1 protein

overexpressing GLC4/*adr* cells).[9] Moreover, these flavonoids efficiently acted as apoptosis-inducing agents.[6,9]

Apoptosis, or programmed cell death, is an important physiologic process in normal development,[12] and apoptosis induction is a key therapeutic strategy for cancer control.[13,14] The major challenge in treating cancer is that many tumor cells carry mutations in central apoptotic genes, such as p53, Bcl family proteins, or those affecting caspase signaling.[15] The Bcl-2 family determines whether a cell lives or dies by controlling the release of mitochondrial apoptogenic factors associated with death proteases called caspases, which in turn induce a cascade of proteolytic cleavage events.[16]

We previously reported that quercetin, Siamois 1, and Siamois 2 induced apoptosis of a breast tumor in xenograft mice when compounds were used at levels similar to physiological concentrations (≤ 20 μM). Moreover, at the range of concentrations used, these compounds do not damage liver tissue.[17] In this study, the cytoxicity of Siamois®, Siamois 1, and Siamois 2 compared to doxorubicin was investigated using normal myoblasts and cancer cell lines. The suspected intracellular targets of Siamois® polyphenols were visualized and analyzed by staining living cells with an equimolar cocktail of acridine orange, rhodamine B and pirarubicin. The polyphenols promoted cell growth of normal myoblasts, while doxorubicin (500 nM) completely inhibited cell growth. Siamois 1 and Siamois 2 had similar anticancer efficacy and induced mitochondrial permiabilization and apoptosis of human breast carcinoma MDA-MB 435, human erythromyelogenous leukemic drug-sensitive K562 and drug-resistant K562/*adr* cells, and human small cell lung carcinoma drug-sensitive GLC4 and drug-resistant GLC4/*adr* cells.

Materials and Methods

Drugs and chemicals

Lyophilized Siamois®, Siamois 1 and Siamois 2 were prepared in house and the polyphenolic content was standardized as described by Dechsupa *et al*.[17] Adriamycin and doxorubicin were purchased from Sigma (USA). Adriamycin stock solution was prepared in double distilled water just before use. Stock solutions were diluted in water to approximately 10 µM, and concentrations were spectrophotometrically determined (ε_{480} = 11,500 $M^{-1}.cm^{-1}$). All experiments were performed using HEPES-Na^{+} buffer (20 mM HEPES buffer plus 132 mM NaCl, 3.5 mM KCl, 1 mM $CaCl_2$, and 1.5 mM $MgCl_2$, pH 7.25 at 37 °C).

Establshment of primary human myoblasts cultures

Under local anesthesia, a human biopsy sample was taken of the thigh *vastus lateralis* through a 1-cm incision. Local anesthetic agents appear to stimulate dormant myogenic cells. The sample was then immersed in complete RPMI 1640 (GIBCO BRL, USA) culture medium and stored at 4 ˚C. Cell isolation and culturing procedures were started within 10 h of biopsy.

To harvest human myoblasts, adipose tissue and fascia were first removed and the muscle was carefully minced with scissors in a biohazard cabinet using an aseptic technique. The muscle pieces were washed with RPMI 1640, incubated in 5 mL calcium and magnesium free Hank's balanced salt solution (HBSS) containing 20 $mg.mL^{-1}$ amphotericin B (ABBOTT France SA, France) for 20 minutes, washed again with RPMI 1640, incubated in 5 mL HBSS containing 10000 $U.mL^{-1}$ and 10000 $mg.mL^{-1}$ penicillin/streptomycin (Biochrom AG, Berlin, Germany), and then washed once more with RPMI 1640. The pieces were mechanically minced using a surgical blade and then digested using collagenase (20 $mg.mL^{-1}$, Biochrom AG, Berlin, Germany) at 37 ˚C for 30

minutes. Ten mL of RPMI 1640 was then added to stop collagenase digestion. The sample was centrifuged, and the pellets were washed once with RPMI 1640, resuspended in 1 mL HBSS containing 0.25% trypsin-EDTA (GIBCO BRL, USA), and then incubated at 37 ˚C for 30 minutes. Trypsin digestion was stopped by the addition of fresh RPMI 1640 (5 mL). The sample was then centrifuged and the pellets were washed twice with RPMI 1640. The cells were resuspended in RPMI 1640 supplemented with 20 % fetal calf serum (GIBCO BRL, USA) and 1 % penicillin/streptomycin (Biochrom AG, Berlin, Germany) and plated in a culture flask coated with 0.2% gelatin. Cells were grown in a CO_2-incubator at 37 °C at 5 % CO_2 and 95% humidify for approximately six weeks, or until the cultures reached approximately 70% confluence. The cells were harvested by trypsinization (2 mL 0.25% trypsin-EDTA) and passaged at a 1:10 dilution every six weeks. All experiments were performed using cells that had been passaged ≤ 5 times.

Detection of desmin and CD56 co-expression

To identify myoblasts, cells were stained for co-expression of desmin and CD56.[18,19] Cell culture flasks were periodically observed using an inverted light microsope and an inverted fluorescence microscope (Nikon model TE-2000E) equipped with a filter box model B-2E/C coupled to a Nikon digital camera, model DXM 1200F. When the cells reached subconfluency, they were harvested by trypsinization and 10^4 cells were resuspended in 500 µL of a phosphate-buffered solution in the presence of 10 µL CD56-PE (Immunotech, a Coulter Company), the myocyte-associated neural cell adhesion molecule (NCAM/CD56)[18]. Cells were incubated in the dark at 25˚C for 30 minutes prior to the addition of paraformaldehyde [500 µL phosphate-buffered solution containing 1% (v/v) formaldehyde], and cells were then washed in PBS. The fixed cells were incubated for 30 min at 4°C with a FITC-conjugated mouse anti-desmin antibody

(clone D33; Dako Corp) at 2.5 mg.mL^{-1} in PBS containing 0.1% saponin and 10% FBS. The immunolabeled cells were then washed, incubated 30 min at 4°C, and analyzed by flow cytometry. After 3 passages, cells at 70 % confluence demonstrated a well organized, mussel tissue like lining, and 51 ± 3% of the cell population co-expressed the two myoblast markers CD56 and desmin.

Fluorescence micrographs of living cells

Cells were cultured in RPMI 1640 supplemented with 20% fetal bovine serum (Gibco Biocult Ltd.) and 1% penicillin-streptomycin. Cell cultures were started with 10^5 cell.mL^{-1} and allowed to grow to approximately 70% confluence. The culture medium was removed, and cells were rinsed once with HEPES-Na^+ buffer pH 7.25. A mixture of acridine orange, a specific marker of lysosomes and cytoskeleton proteins[20,21], rhodamine B, a specific mitochondrial dye[22], and pirarubicin, a nuclear compartment marker[23] was then added to the cells (1 μM final concentration). The sample was incubated at 37˚C for 30 minutes prior to examination with an inverted fluorescence microscope (Nikon model TE-2000E) equipped with a filter box model B-2E/C coupled to a Nikon digital camera, model DXM 1200F.

Cell lines, cell culture and cytotoxicity assay

The K562 human erythromyelogenous leukemia cell line and its DOX-resistant, P-glycoprotein-overexpressing K562/*adr* subline[24,25], and the GLC4 human small cell lung carcinoma cell line and its DOX-resistant, MRP1-overexpressing GLC4/*adr* subline[22] were routinely cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum in a humidified atmosphere with 5% CO_2 at 37 °C. For the cytotoxicity assays, cells were plated at a density at 5×10^5 cell.mL^{-1} and used 24 h later (8×10^5 cell.mL^{-1})

so that cells were in exponential growth phase. Cell viability was assessed by trypan blue exclusion, and cell number was determined with a haemocytometer.

MDA-MB 435 is an estrogen receptor-negative cell line isolated from the pleural effusion of a patient with breast carcinomas.[26] The cells were routinely cultured in RPMI 1640 medium containing 0.3 $g.L^{-1}$ L-glutamine and supplemented with 10% fetal calf serum, 2 mM pyruvate, 100 $U.mL^{-1}$ penicillin, and 100 $\mu g.mL^{-1}$ streptomycin (all supplements purchased from Life Technology, Inc.) in a humidified incubator at 37 °C in 5% CO_2. Cells were subcultured twice a week. Prior to all experiments, the cells were trypsinized (0.05 % trypsin, 0.02% ethelenediaminetetra acetic acid, EDTA) and resuspended at a density of 5×10^5 $cell.mL^{-1}$ so that cells were in an exponential growth phase when used 24 h later (8×10^5 $cell.mL^{-1}$).

Cytotoxicity of Siamois®, Siamois 1 and Siamois 2 in normal myoblasts

Myoblasts (10^4 cells) were seeded into 0.2% gelatine coated six well plates containing 4 mL RPMI 1640 supplemented with 20 % fetal calf serum and 1 % penicillin/streptomycin (BioMedia). The cells were incubated at 37 °C in 5 % CO_2 and 95% humidify for one week to allow the viable myocytes to attach to the gelatin. When the cell density in the wells reached 10^5 $cell.mL^{-1}$, compounds ranging in concentration from 0 to 0.5 $mg.mL^{-1}$ were added. Cell viability was then determined using an MTT assay based on the reduction of MTT to purple-colored formazan by live cells. The percentage viability was determined at 72 h and was plotted versus the identity of the compound.

Cytotoxicity of Siamois®, Siamois 1 and Siamois 2 against cancer cells

Cells (5×10^4 mL^{-1}) were incubated in the presence of various concentrations of drugs. Cells viability was then determined using a MTT assay as described above. The

concentration of drug required to inhibit cell growth by 50% at 72 h (IC_{50}) was determined by plotting the percentage of cell growth inhibition versus the drug concentration. The resistance factor (RF) was defined as the IC_{50} of resistant cells divided by the IC_{50} of the corresponding sensitive cells.[21,22] Under our experimental conditions, the IC_{50} values were 10 ± 2 nM for K562 and GLC4 cells, and the RF values were 40 and 7 for K562/*adr* and GLC4/*adr* cells, respectively.

Induction of apoptosis

For the apoptosis assay, exponentially dividing cells were seeded in a T25 fastk at an initial density at 10^5 in 5 mL medium. After 24 h, various compound concentrations ranging from 0 to 0.2 $mg.mL^{-1}$ were added and the cells were further incubated at 37 °C for 0.5, 1, 3, 6, 18, and 24 h. Different concentrations of anti-human CD95/Fas/TNFRSF6 antibody MAB142 (R&D Systems Inc.) ranging from 2.5 to 15 $\mu g.mL^{-1}$ were used as positive controls to induce apoptosis; these concentrations were ten fold higher than that reported by Yu *et al*.[27]

Cytofluorometric staining of the cells

To measure the level of apoptosis 10^6 Cells were centrifuged for 5 min at 1000×g at room temperature (18-24 ^{0}C), resuspended, and washed once with 5 mL phosphate-buffered saline prior to staining with Annexin V (apoptosis detection kit, R&D Systems). Flow cytometry analysis was performed using a Coulter Epics XL-MCL (Coultronics France SA), and 5000 events per sample were evaluated. Biparametric histograms were used to visualise cells distribution as a function of signal intensity with respect to Annexin V-FITC and Propidium iodide.

Determination of intracellular ATP, ADP and AMP levels

Cells (10^6 mL^{-1}) were incubated in the presence of various concentrations of Siamois® at 37 °C in 5 % CO_2 and 95% humidity for 30 minutes. The cells were collected and washed once using HEPES-Na^+ pH 7.3. Cell extraction was performed at 4°C using 500 µL lysis solution containing 0.6 M $HClO_4$. The samples were vigorously mixed for 5 minutes, centrifuged at 3000g for 10 minutes, and the perchoric residue was collected. The supernatant was precipitated by the addition of 2 M KOH (120 µL), and then the $KClO_4$ was removed following centrifugation at 3000g for 10 min. The sample was passed through a 0.45 µm filter before HPLC analysis [Shimadzu (SPD-M20A photodiode Array detector, LC-20AD parallel type double plunger pump unit]. Analytical RP-HPLC chromatography was performed on an Innertsil-ODS-3, C_8, 5 µm particle size, 250 x 4.6 mm i.d. column (GL Sciences Inc.) protected with a guard column of the same material [20 mm x 2.1 mm, 3 µm packing (GL Sciences Inc.)]. The solvent system was 50 mM NaH_2PO_4 pH 7.0 at flow rate 1 $mL.min^{-1}$.

Results

Fluorescence micrographs of intracellular organelles and cellular function

Primary myoblasts cultures were established and initially consisted of cells with various morphologies. The cells preserved their proliferative and differentiation properties, and after propagation through 3 passages, cells at 70 % confluence demonstrated a well organized, muscle-like lining. Intracellular organelles, including lysosomes, nuclei, and mitochondria, were clearly visualized in living cells using acridine orange, rhodamine B, and pirarubicin (Figure 1). These dyes allowed clear observation of: (1) bright green fluorescence resulting from the interaction of acridine orange with cytoskeleton proteins; (2) reddish orange fluorescence resulting from the accumulation of acridine orange in lysosomes; (3) bright yellow fluorescence resulting from rhodamine B

accumulation in energetic mitochondria; and (4) dim red fluorescence resulting from pirarubicin accumulation in the nucleus. The reddish orange fluorescence completely disappeared in the presence of 3 μM monensin, which results in the successive elimination of lysosome pH gradients. Similarly, bright yellow fluorescence levels were observed in the presence of 7 μM FCCP and 10 μM oligomycin. However, the yellow fluorescence was recovered in the presence of 5 μM cyclosporine A. FCCP, a protonophore, is an uncloupling agent that induces depolization of mitochondria by dissipating the H^+ electrochemical gradient across the inner membrane, while cyclosporine A is specific inhibitor of the permeability transition pore, which induces repolarization of mitochondria.[23] An increase in bright yellow fluorescence intensity was observed when cells were incubated with 5 μM cyclosporine A.

Myoblasts were also treated with 500 μg/mL of Siamois® and 5 $\mu g.mL^{-1}$ Siamois 1 or Siamois 2 for 72 h and visualized using fluorescence microscopy (Figure 1b and c, respectively). The micrographs revealed that the cultures consisted of cells with various morphologies and that their proliferative and differentiation properties were preserved. All compounds caused a decrease in reddish orange and bright yellow fluorescence intensity and reduced the organelle density compared to untreated cells. Treatment with the control compound, doxorubicin (500 nM), also decreased reddish orange and bright yellow fluorescence intensity and the density of organelles compared to untreated cells (Figure 1d). However, in contrast to doxorubicin, concentrations of Siamois®, Siamois 1 and Siamois 2 ranging from 0.05 $mg.mL^{-1}$ to 0.5 $mg.mL^{-1}$ efficiently promoted cell growth (Figure 2a). Moreover, Siamois® (up to 500 $\mu g.mL^{-1}$), and Siamois 1 and 2 (5 $\mu g.mL^{-1}$) did not adversely affect cell viability even at high concentrations (Figure 2b).

Anticancer activity

Siamois® slightly inhibited cancer cell proliferation, but Siamois 2 exhibited significant anticancer action against MDA-MB 435, GLC4, GLC4/*adr*, K562 and K562/*adr* cells (Figure 3). The Siamois 2 concentration required to inhibit 50% of cell growth (IC_{50} value) was 2.2 ± 0.73 $mg.mL^{-1}$ for MDA-MB 435, 3.28 ± 0.38 $mg.mL^{-1}$ for K562 and K562/*adr*, and 3.2 ± 0.2 $mg.mL^{-1}$for GLC4 and GLC4/*adr* cells. Siamois 1 exhibited about 30 to 100 fold greater anticancer activity than Siamois 2 against the five cancer cell lines (Figure 3). IC_{50} values were 0.05 ± 0.01 $mg.mL^{-1}$ for MDA-MB 435, 0.12 ± 0.02 $mg.mL^{-1}$ for K562 and K562/*adr*, and 0.18 ± 0.02 $mg.mL^{-1}$ for GLC4 and 0.10 ± 0.2 $mg.mL^{-1}$ GLC4/*adr* cells.

Intracellular ATP, ADP and AMP levels

In order to verify the dominant intracellular targets of the tested compounds, two series of experiments were performed in parallel. The cancer cells were incubated in the presence of the compounds for 24 h at 37 °C in a humidified incubator at 5% CO_2. In one series of experiments, the treated cells were examined under an inverted fluorescence microscope. Siamois 1 and Siamois 2 caused a dramatic decrease in bright yellow fluorescence intensity and the density of organelles, reflecting an alteration in mitochondrial function. In a second experimental series, the cells were collected for analysis of cellular ATP. Typical results of the variation in ATP, ADP and AMP levels in cancer cells treated with Siamois 2 are shown in Figure 4. These data clearly demonstrate that Siamois 2 caused a dramatic decrease in the amount of ATP, ADP and AMP. Similar results were obtained for Siamois 1. Thus, both compounds affected energy levels within the cells.

Apoptosis-inducing activity

The ability of Siamois 1 and Siamois 2 to induce apoptosis was evaluated by Annexin V staining. Typical histograms of Annexin V-FITC binding to

phosphatidylserine (PS) on apoptotic MDA-MB 435 cells incubated with the control CD95/Fas/TNFRSF6 antibody MAB142, Siamois 1, or Siamois 2 are shown in Figure 5. The total number of apoptotic cells was determined from the number of cells found in quadrant 2 and 4. Cells in quadrant 4 (PI negative and Annexin V-FITC positive) are at an early stage of apoptotsis, while quadrant 2 cells (PI and Annexin V-FITC positive) are at a late apoptotic stage. In the absence of treatment, the basal level of apoptotsis in the MDA-MB-435 cell line was 3.2 ± 1.8 %. Siamois 1 and Siamois 2 (up to 200 $\mu g.mL^{-1}$) induced time and concentration dependent apoptosis of MDA-MB-435 cells (Figure 6). Apoptotic cell death was detected within 30 min for all compounds used (Figure 6a), which remained at a steady state until 24 h. The total percentage of apoptotic cells (%) was determined at 6 h as indicated in Figure 6b. Similar experiments were performed using K562, K562/*adr*, GLC4, and GLC4/*adr* cells. Siamois® induced comparable levels of apoptosis in all four cell lines, as indicated in Figure 7, and apoptosis levels appeared to be independent of the time the wine was aged (50% total apoptosis at 15 $mg.mL^{-1}$ for 24 h).

Discussion

Live cell imaging is a very useful tool for understanding cellular responses to stimuli, particularly when monitoring the interaction of drugs with specific intracellular organelles. This study showed for the first time that live cell imaging can be used to determine intracellular organelle function by staining the cells with an equimolar cocktail of acridine orange, rhodamine B and pirarubicin prior to analysis using an inverted fluorescence microscope. Imaging was conducted at 37°C using a circulating water bath system, allowing cellular functions to be visualized under physiological conditions. Indeed, this system allowed us to clearly visualize; (1) the bright green fluorescence

resulting from the interaction of acridine orange with cytoskeleton proteins, (2) reddish orange fluorescence resulting from accumulation of acridine orange in lysosomes, (3) bright yellow fluorescence resulting from rhodamine B accumulation in the energetic mitochondria, and (4) dim red fluorescence due to pirarubicin accumulation in the nucleus. The changes in fluorescence intensity (e.g. rhodamine B) and the density of organelles corresponded to changes in the cellular levels of ATP, ADP and AMP. These changes also correlated well with apoptosis induction. Indeed, since we could image mitochondrial function of living cells under specific treatment conditions, we can now consider a decrease in rhodamine B fluorescence intensity and number as potential early markers of apoptosis.

Siamois® polyphenols efficiently inhibited cell growth of 5 cancer cell lines, (MDA-MB-435, K562, K562/*adr*, GLC4 and GLC4/*adr* cells) with similar efficacy. Even in drug-resistant sublines, such as K562/*adr,* which overexpresses P-gp, and GLC4/*adr,* which overexpresses MRP1 protein, Siamois® polyphenols had similar cytotoxic activity. This suggests that Siamois® polyphenols stimulate collateral sensitivity of MDR cells. Our results further showed that Siamois® polyphenols efficiently inducted apoptosis in cancer cell used in this study, although the time that the red wine was aged did not appear to affect this activity. Moreover, the live cell imaging data clearly demonstrated that Siamois® polyphenols impair mitochondrial function in both myoblasts and drug-sensitive and –resistant cancer cells. We previously reported that in similar experimental conditions, a decrease in cellular ATP levels was associated with a decrease in mitochondrial membrane potential, resulting in apoptosis of cancer cells.[23,28] Together with our current data, these results suggest that the action of these polyphenols on cancer cells can be described as "assisted suicide". In addition, we previously reported that the antiproliferative and apoptosis-inducing effects of quercetin,

Siamios 1, and Siamois 2 on MDA-MB 435 cells *in vitro* were effectively extrapolated *in vivo*.[17]

In contrast to its proapoptotic activity in cancer cell lines, Siamois® polyphenols did not cause deleterious effects in myoblasts. Rather, they promoted normal myoblasts growth. Since Siamois® affected mitochondrial function of both normal myoblasts and cancer cells but only caused dramatic depolarization of cancer cell mitochondria, we hypothesize that normal cells may more efficiently maintain their mitochondrial energetic state. Thus, during carcinogenesis, cancer cells may loose these systems, causing changes in cellular physiology that increase their sensitivity to drugs that affect cellular energy balance.[29] These data are in line with numerous reports that suggest that red wine polyphenols are non toxic and have few side effects. [30,31]

In conclusion, the use of an inverted microscope and an equimolar cocktail of acridine orange, rhodamine B and pirarubicin in living cells allows clear visualization of the intracellular targets of Siamois® polyphenols. Using this technique together with biophysical analysis of cellular ATP, ADP and AMP content, the number of apoptotic cells, and spontaneous changes in the mitochondrial membrane potential, clearly showed that Siamois® promotes normal myoblast growth but efficiently inhibits proliferation and induces apoptosis of cancer cells at the mitochondrial level. These methods are thus suitable for visualizing the intracellular targets of anticancer molecules whose mechanism of action may involve mitochondria.

Acknowledgements

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Figure Captions

Figure 1. Fluorescence micrograph of untreated myoblasts (a), or myoblasts treated with 0.5 mg.mL^{-1} Siamois 1 (b), Siamois 2 (c), or 500 nM doxorubicin (d).

Following the 3rd passage, cells were seeded into 0.2% gelatine coated six well plates containing 2 mL RPMI 1640 supplemented with 20 % fetal calf serum and 1 % penicillin/streptomycin. Cells (10^5 mL^{-1}/ well) were incubated at 37 °C in 5 % CO_2 and 95% humidity for one week to allow the viable myocytes to attach. Compounds were added at the indicated concentrations, and the cells were incubated for an additional 72 h. An equimolar cocktail of acridine orange, rhodamine B and pirarubicin was added prior to placement on the inverted fluorescence microscope.

Figure 2. Effects of Siamois®, Siamois 1 and Siamois 2 (a) and doxorubicin (b) on myoblast growth.

Cells (10^4 mL^{-1}) were seeded into 0.2% gelatine coated six well plates containing 2 mL RPMI 1640 supplemented with 20 % fetal calf serum and 1 % penicillin/streptomycin. The cells (10^5 mL^{-1}/ well) were incubated at 37 °C in 5 % CO_2 and 95% humidity for an additional week to allow the viable myocytes to attach. Compounds were added at indicated concentrations, and cell viability was determined using an MTT assay based on the reduction of MTT to purple-colored formazan by live cells. Results represent the averages of triplicate cultures.

Figure 3. Effects of Siamois 1 on cell growth of K562, K562/*adr* and MDA-MB 435 (a) and GLC4 and GLC4/*adr* cells (b), and the effects of Siamois 2 on K562 and K562/*adr* (c) and GLC4 and GLC4/*adr* cells (d). Cells (5×10^4 mL^{-1}) were incubated in the presence

of the indicated drug concentrations. At 72 h cell viability was determined using an MTT assay. The concentration of the drug required to inhibit cell growth by 50% (IC_{50}) was determined. Results represent the average of triplicate cultures. Error bars identify the range of values.

Figure 4 Effects of Siamois® on cellular ATP (a), ADP (b) and AMP (c) levels.

Cells (10^6 mL^{-1}) were incubated in the presence of the indicated concentration of Siamois® in an incubator at 37 °C in 5 % CO_2 and 95% humidity for 30 minutes. The cells were collected and washed once with HEPES-Na^+ pH 7.3. Cellular extraction was performed at 4°C using 500 µL lysis solution containing 0.6 M $HClO_4$. The samples were vigorously mixed for 5 minutes, centrifuged at 3000xg for 10 minutes, and the perchloric residue was collected. The supernatant was precipitated by the addition of 2 M KOH (120 µL) and then the $KClO_4$ was removed by centrifugation at 3000xg for 10 min. The sample was passed through a 0.45 µm filter before HPLC analysis. Results represent the average of triplicate cultures.

Figure 5. Representative biparametric histrograms of Annexin V-FITC versus PI staining of untreated (a) and treated (b) MDA-MB 435 cells after a 6 h incubation with 5 $\mu g.mL^{-1}$ anti-CD95, (c) 60 $\mu g.mL^{-1}$ Siamois 1, and (d) 60 $\mu g.mL^{-1}$ Siamois® as described in the Materials and Methods.

Figure 6. Apoptosis inducing activities of Siamois 1 and Siamois 2 in MDA-MB 435 cells. Total apoptosis as a function of time (a) and drug concentration (b).

Cells were exposed to Siamois 1 and Siamois 2 at various concentrations and incubated for the indicted times before staining with Annexin V-FITC and PI. Flow cytometry analysis was performed using a Coulter Epics XL-MCL (Coultronics France SA), and 5000 events per sample were evaluated. Each value represents the mean ± S.D. for three independent experiments. ($P< 0.05$)

Figure 7. Apoptosis-inducing activities of Siamois® in (a) K562 and K562/*adr* and (b) GLC4 and GLC4/*adr* cells.

Cells were exposed to 150 $\mu g.mL^{-1}$ Siamois® for 24 h prior to staining with Annexin V-FITC and PI. Flow cytometry analysis was performed using a Coulter Epics XL-MCL (Coultronics France SA), and 5000 events per sample were evaluated.. Each value represents the mean ± S.D. of three independent experiments. ($P< 0.05$ except *)

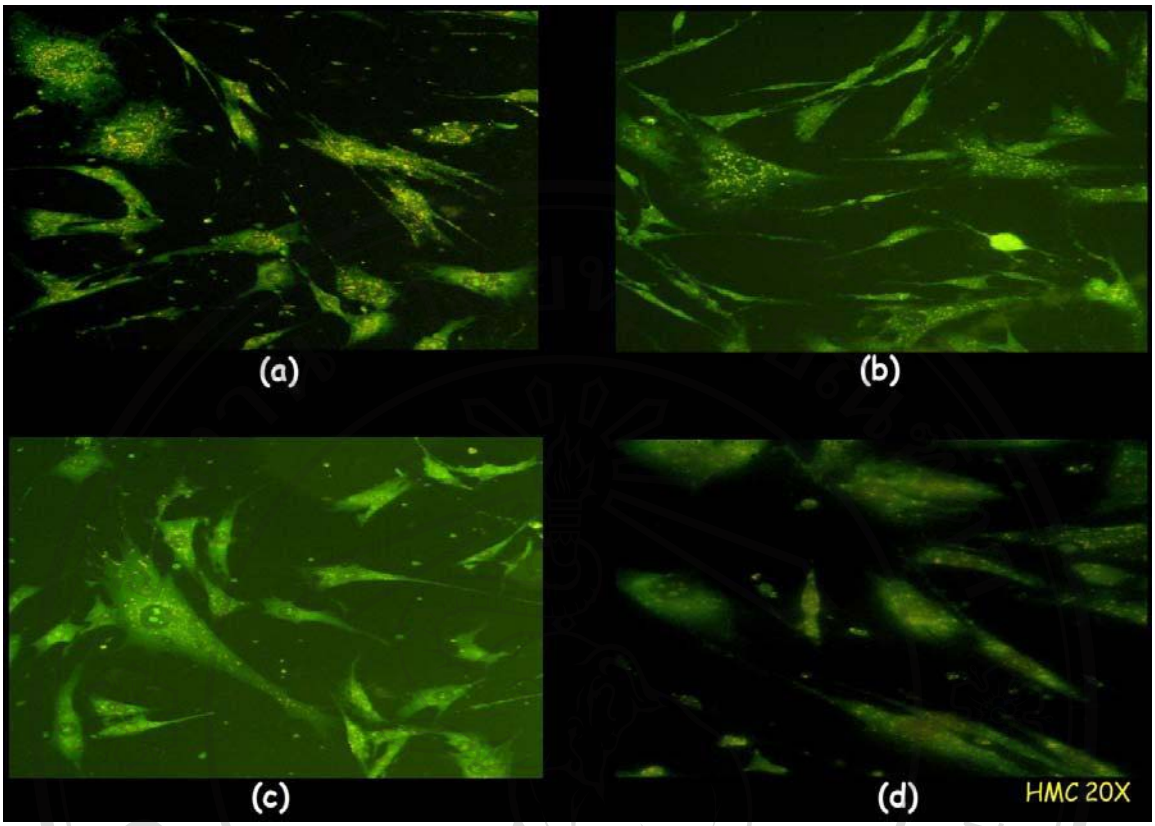


Figure 1

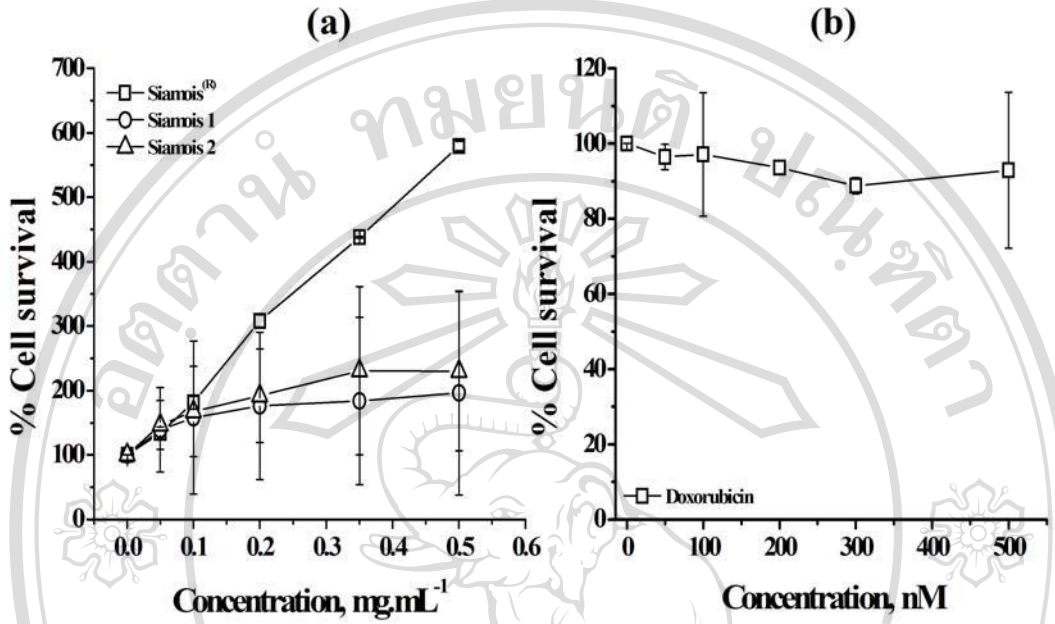
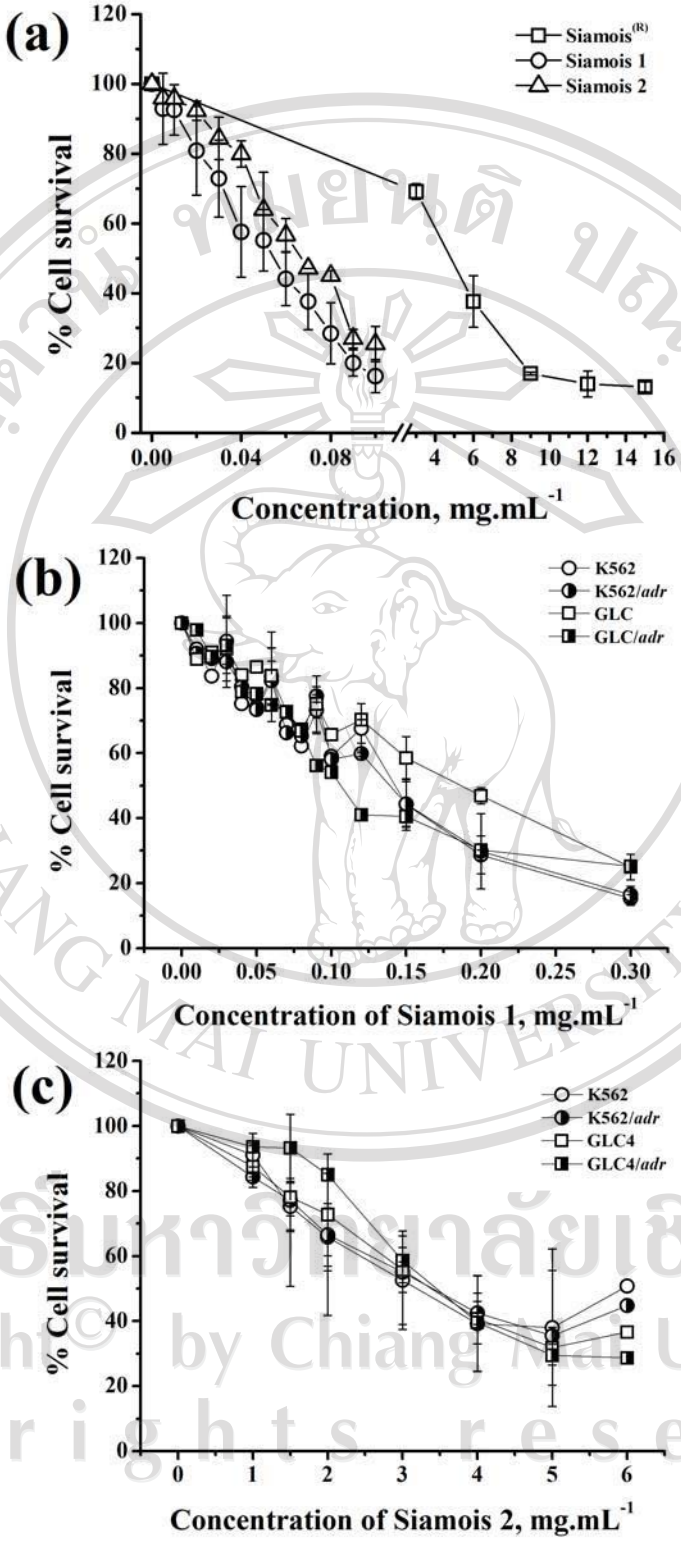


Figure 2



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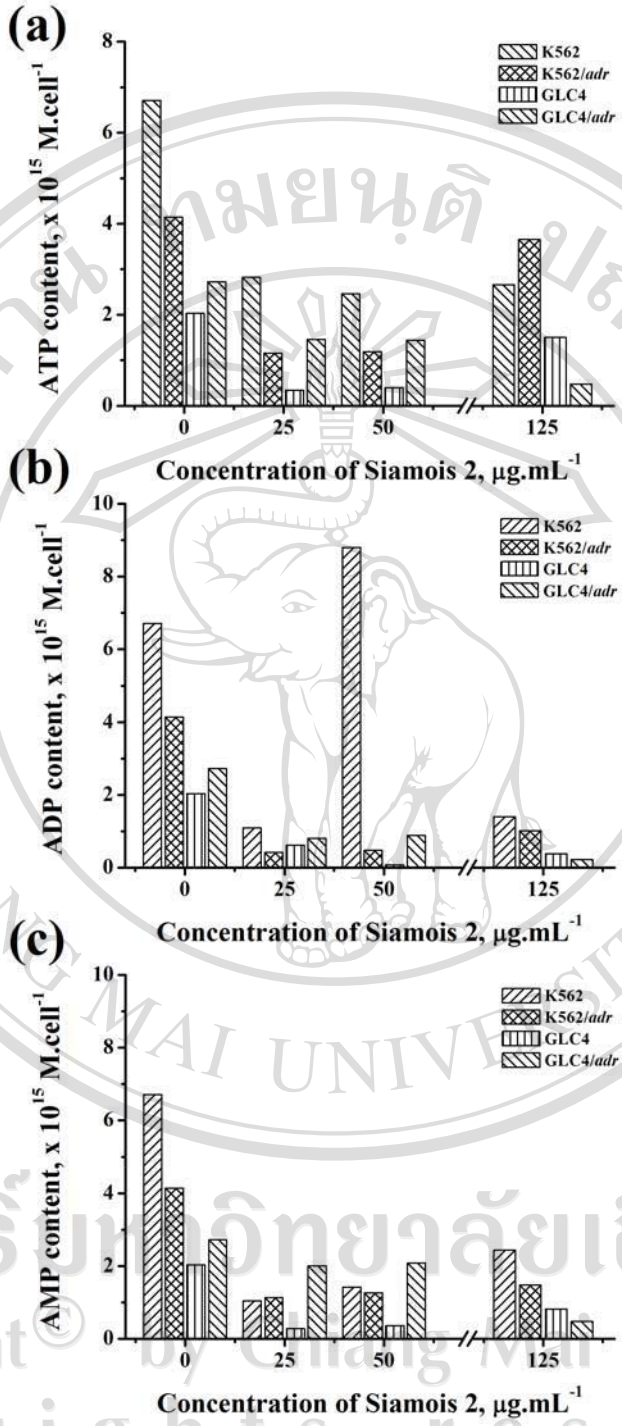

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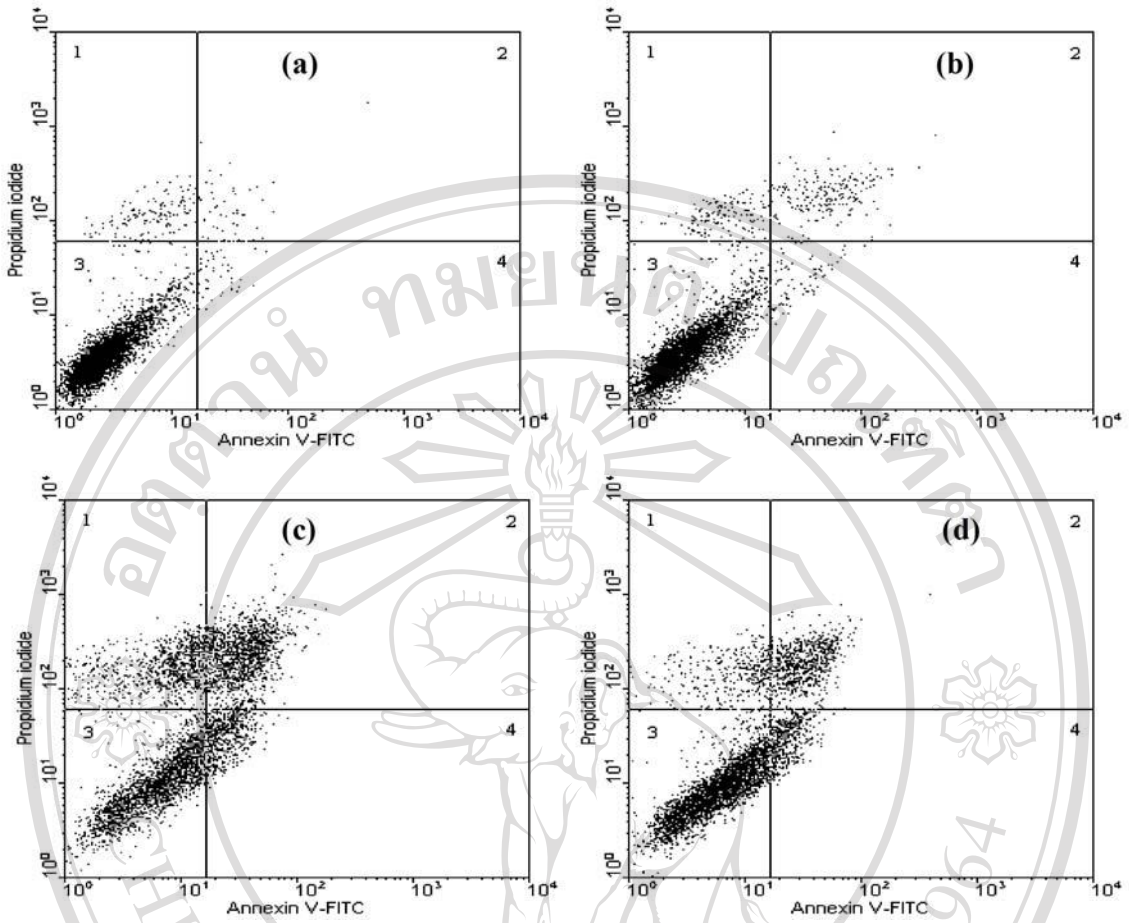

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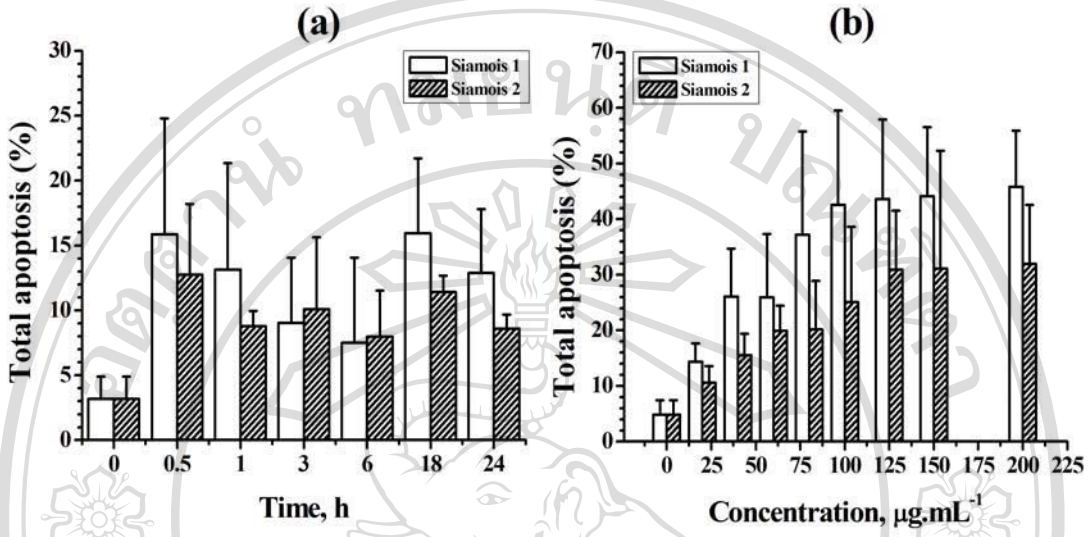

Figure 6

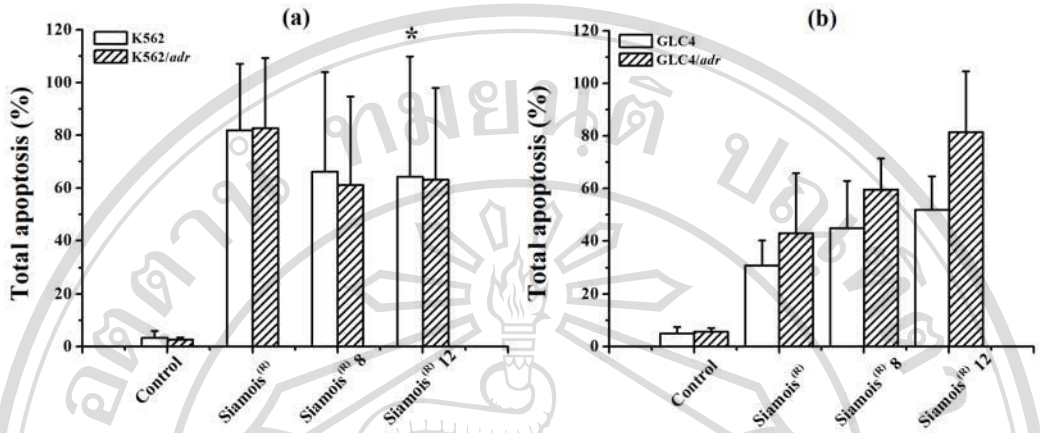

Figure 7

CURRICULUM VITAE

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