

## CHAPTER 5

### DISCUSSION AND CONCLUSION

Anti-cancer drug discovery has focused on natural products to avoid the adverse effects of the clinically used cytotoxic drugs and such anti-cancer compounds should ideally exhibit cytotoxicity against cancer cells via apoptotic induction. This study emphasizes on natural polyphenolic compounds, the so-called Siamois<sup>®</sup> obtained from red wine and Thai medicinal plant, Mamao (*A. thwaitesianum* Müll.Arg.). Siamois<sup>®</sup>, Siamois 1 and Siamois 2 crude extracts efficiently inhibited cell growth of 4 cancer cell lines, (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<sup>®</sup>, Siamois 1 and Siamois 2 crude extracts had similar cytotoxic activity. This suggests that Siamois<sup>®</sup>, Siamois 1 and Siamois 2 crude extracts stimulate collateral sensitivity of MDR cells. Our results further showed that Siamois<sup>®</sup>, Siamois 1 and Siamois 2 crude extracts efficiently induced apoptosis in the cancer cells used in this study, independently of the age of the red wine. Moreover, the live cell imaging data demonstrated that Siamois<sup>®</sup>, Siamois 1 and Siamois 2 crude extracts impaired 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 (15, 185). 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, Siamois 1, and Siamois 2 on MDA-MB 435 cells observed *in vitro* were effectively extrapolated to *in vivo* condition (14).

In contrast to its pro-apoptotic activity in cancer cell lines, Siamois<sup>®</sup>, Siamois 1 and Siamois 2 crude extracts did not cause deleterious effects on myoblasts. Rather, they promoted normal myoblasts growth. Since Siamois<sup>®</sup> 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 lose these systems, causing changes in cellular physiology that increase their sensitivity to drugs that affect cellular energy balance. These data are in line with numerous reports that suggest that red wine polyphenols are non toxic and have few side effects (207, 208).

Living 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 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. It should be noted that an increase in bright yellow fluorescence intensity was found in the presence of 5  $\mu\text{M}$  cyclosporine A. FCCP is a protonophore, an uncloupling agent that induced depolarization of mitochondria by dissipating the  $\text{H}^+$  electrochemical gradient across the inner membrane while cyclosporine A is a specific inhibitor of the permeability transition pore, which induces repolarization of mitochondria (23). 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 their number as potential early markers of apoptosis.

The fluorescence micrographs using inverted microscope combined with equimolar cocktail of acridine orange, rhodamine B and pirarubicin of living cells are very useful for visualizing the intracellular targets of Siamois<sup>®</sup>, Siamois 1 and Siamois 2 crude extracts. The Siamois<sup>®</sup> promoted normal myoblast growth, but efficiently inhibited proliferation and induced apoptosis of cancer cells at the mitochondrial level. These methods are suitable for visualizing the intracellular targets of anti-cancer molecules that are suspected to mediate action at the mitochondria. However, the biophysical analysis of ATP, ADP and AMP content, the number of apoptotic cells and the spontaneous changes of the mitochondrial membrane potential were recommended in order to identify the mechanisms of the anti-cancer action.

As shown in the characterization of Siamois<sup>®</sup>, Siamois 1, and Siamois 2 crude extracts, flavonoids, such as resveratrol, quercetin, eriodictyol and apigenin were characterized and considered as active molecules. The effect of quercetin on the mitochondrial energetic state was determined in this work. At a quercetin concentration

of 10  $\mu\text{M}$ , the spontaneous change in  $|\Delta\Psi_m|$  indicated the energetic state of mitochondria and cells could be induced. An increase followed by a decrease in  $|\Delta\Psi_m|$  value was associated with an induction of apoptosis that could be detected after 1 h of incubation with quercetin. Our results are in an agreement with other studies, which reported that quercetin (60  $\mu\text{M}$ ) and other flavonoids induced apoptosis in HL-60 cells by releasing cytochrome c and inducing caspase-9 processing (84, 85). The  $|\Delta\Psi_m|$  of K562 and K562/Adr cells is equal to  $160 \pm 1.0$  mV and  $145 \pm 1.2$  mV, respectively. Our previous work showed that artemisinin and its derivatives reversed MDR phenomenon at the mitochondrial level (29). The correlation between the impairment of mitochondrial energetic state (decreasing in  $|\Delta\Psi_m|$  value) and an induction of apoptosis (the percentage of early apoptotic cells) by quercetin can be observed. Furthermore, the previous study suggested that at higher concentrations of quercetin, the  $|\Delta\Psi_m|$  decreases in very narrow range, whereas the percentage of early apoptotic cells increased in greater degree of range (15). The results of this study reveal that quercetin provokes its cytotoxicity at the mitochondria level, impairing mitochondrial energetic state and then inducing apoptosis and inhibition of cancer cell proliferation. Quercetin, therefore, might be an active constituent of Siamois<sup>®</sup>, Siamois 1, Siamois 2, particularly for overcoming MDR phenomena.

Extensive studies indicated that both hyperactivated NF $\kappa$ B and overexpression of multidrug transporters play important roles in cancer chemoresistance (107, 149, 194, 200, 209, 210). Since expression of the multidrug transporter P-gp was found to be NF $\kappa$ B-dependent, it is believed that NF $\kappa$ B inhibitors can decrease P-gp expression and restore chemosensitivity (156, 166). 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 1 and Siamois 2 in a xenograft model *in vivo* (14). Here we further analysed whether quercetin, kaempferol, eriodictyol and WP283 which are present in Siamois<sup>®</sup> crude extract hold therapeutic promise as NFκB inhibitors for chemosensitization of doxorubicin-resistant K562/Adr erythromyelogenous leukemic cells. In NFκB reporter gene studies, we measured dose-dependent repression of IL-6 with quercetin, kaempferol, eriodictyol and WP283 with IC<sub>50</sub> values in the range of 0.1-50 μM, respectively. Furthermore, upon comparing endogenous gene expression of NFκB target genes, we observe similar potencies in NFκB-dependent gene repression by quercetin, kaempferol, eriodictyol and WP283 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, cyclin D1, VEGF and mdrl/P-gp). 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 quercetin, kaempferol, eriodictyol and WP283. Upon characterization of the major NFκB activation and transactivation pathways, we found differential regulation of NFκB activity by quercetin, kaempferol, eriodictyol and WP283. Interestingly, IκBα degradation and NFκB/DNA binding was significantly reduced by all compounds tested in both cell types, among which 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 quercetin, kaempferol, eriodictyol and WP283 in contrast to inhibition of inducible NFκB/DNA-binding. Furthermore, relative

composition of NF $\kappa$ B/DNA-binding complexes reveals that K562 cells contain much higher levels of p65-p65 homodimers. Of particular interest, the inflammatory cytokine IL-8 was found to preferentially bind p65-p65 homodimers instead of p50-p50 and p50-p65 dimers (211), which could explain strong expression of inflammatory cytokines in K562 cells. From another perspective, NF $\kappa$ B dimer composition may also depend on the post-translational modification repertoire present on NF $\kappa$ B (195, 212, 213). More specifically, we found variable compound-specific effects on p38 MAPK, MEK1, Akt kinase pathways, which may also interfere with NF $\kappa$ B 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 NF $\kappa$ B transcription factor activity (214-216). Of special note, as doxorubicin was found to increase Sirt1 HDAC levels (200), 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 to cell-specific phospho-acetylation control of transcription factor-DNA binding and transcriptional activity and may prevent NF $\kappa$ B p65 homodimer formation. In addition to cell specific regulation of NF $\kappa$ B, it can be observed from Figure 28 that also AP1 members (i.e. Fra1, c-jun and jun D) and Nrf2 are differentially expressed in both cell types. As such, we can neither exclude compound-specific kinase effects on these transcription factor families, since various NF $\kappa$ B target genes involved in inflammation, metastasis, angiogenesis and drug resistance are also coregulated by AP1 and Nrf2 (198, 199, 217)

Most surprisingly, although inhibition of NF $\kappa$ B activity in general contributes in chemosensitisation of cancer cells (194, 218), treatment of K562/Adr cells with quercetin,

kaempferol, eriodictyol and WP283 failed to cleave caspase-3 and trigger late apoptosis to similar levels as observed in K562 cells, although efficacy of NF $\kappa$ B inhibition and initiation of early apoptosis by quercetin, kaempferol, eriodictyol and WP283 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 and -8 activation, but not formation of the death-inducing signal complex (219-221). The fact that quercetin, kaempferol, eriodictyol and WP283 are able to completely inhibit NF $\kappa$ B target gene expression, hyperactivate MEK1 and trigger early apoptosis in K562/Adr cells argues against the hypothesis that quercetin, kaempferol, eriodictyol and WP283 may not be completely 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 (222). 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 (223).

In conclusion, we found that transcriptional inhibition of NF $\kappa$ B-, 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. However, quercetin, kaempferol, eriodictyol and WP283 may have therapeutic 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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