CHAPTER 2

LITERATURE REVIEWS

1. Evaluation of non-cytotoxic polyphenols as putative new drugs in cancer chemotherapy

The strategies to overcome cancers have evolved since the discovery of cytotoxic agents that killed the cancer cells, to the development of molecules that mediated action at specific biochemical pathway or specific targets. For example, new strategies inspired from molecular biological and biotechnological study. The discovery of oncogenes and the elucidation of their function in the regulation of cell growth were recently proposed as new targets for cancer treatments. However, these targets will have susceptible counterparts involved in normal cell function. Thus, development of strategies to exploit the differences in the oncogene activities in normal and neoplastic cells is still necessary. Indeed, in such strategy, the cytotoxic drugs could be selectively delivered and interact with those intracellular targets for example by direct conjugation to a tumor-specific antibody, or such antibodies could be incorporated into the surface of drug-loaded liposomes.

Programmed cell death, particularly adhesion-dependent regulation of cell survival and apoptosis, is recognized as one of the main homeostatic mechanisms designed to control cell positioning, eliminate misplaced cells and block metastatic dissemination. Numerous studies reported that cancer cells were higher resistant to programmed cell death compared to normal cells. The strategies, focusing on the

mechanism of cell death, were proposed for overcoming cancer diseases by developing a strategy for combination chemotherapy, focused on enhancing the mechanisms by which chemotherapeutic agents kill cells. This is the original idea of a new generation anti-cancer.

A number of drugs used in cancer chemotherapy induce oxidative stress by generation of reactive oxygen species (ROS). Recent studies suggested that ROS formation caused by these drugs might be an alternative mechanism for their cytotoxic effect via inducing apoptosis. Since ROS was proposed to play a role in drug-induced apoptosis, therefore, it is expected that anti-oxidants might inhibit the ability of chemotherapeutic drugs to induce ROS generation. Indeed, the anti-oxidant molecules such as quercetin, kaempferol, apigenin and eriodyctiol strongly exhibited antiproliferation of cancer cells via apoptosis induction at the mitochondrial level (14, 15). Moreover, Choiprasert W. clearly demonstrated that the anti-oxidant molecules, including quercetin and its glycoside derivatives, strongly enhanced pirarubicin cytotoxicity against K562/Adr cells wich overexpress P-glycoprotein (P-gp) and GLC4/Adr cells which overexpress multidrug resistance associated-protein 1 (MRP 1) (16). This indicated that independently to their anti-oxidant activity, these compounds should exihibit a specific action, such as inhibition of the P-gp or MRP 1, and might interact with specific intracellular targets of apoptosis.

Chemopreventive approaches using non-toxic agents aimed at both minimizing ROS formation and inducing apoptosis in tumor cells seem attractive. In a recent study it was demonstrated that novel catechin derivatives obtained from grape procyanidins scavenge free radicals, and reduce cell viability in A375 and M21 melanoma cells (17). In particular, 4b-(S-cysteinyl) epicatechin 3-O-gallate possesses a free radical scavenging capacity and causes a significant S-phase cell-cycle arrest in both cell lines at

concentrations higher than 100 μM. The gallate derivative also induces apoptosis in melanoma cells triggering nuclear condensation and fragmentation, which is confirmed by DNA laddering. In contrast, it does not induce apoptosis in human cancer keratinocytes (HaCaT) (17).

An impressive body of information exists on the antitumor action of plant flavonoids (18). *In vitro* work has concentrated on the direct and indirect actions of flavonoids on tumor cells and has found a variety of anti-cancer effects such as inhibition of cell growth and kinase activity, apoptosis induction, suppression of the secretion of matrix metalloproteinases which are responsible for a tumor-invasive behavior. Furthermore, some studies have reported the impairment of *in vivo* angiogenesis by dietary flavonoids. Experimental animal studies indicated that certain dietary flavonoids possessed antitumor activity. As previously mentionned, the flavonoids are potentially pharmaceutical molecules as a new generation of anti-cancer drugs and many works have attempted to clarify the action of flavonoids at both the cellular and animal level, but the mechanisms underlying the potential anti-cancer action are waiting for further elucidation.

Let's consider the existing therapies: most drugs have been developed largely against the bulk population of tumour cells. The shrinkage of tumour mainly reflects an ability to kill these cells. There are recent studies reporting that the normal stem cells from various tissues tend to be more resistant to chemotherapeutics than mature cell types of the same tissues (19). The reasons for this are not clear, but may relate to high levels of expression of anti-apoptotic proteins (20-23) or ABC transporters, such as the multidrug resistance gene (24, 25). If the same hold true for cancer stem cells, then one would expect that these cancer stem cells would be more resistant to chemotherapeutics than tumour cells with a limited proliferative potential. Even therapies that cause complete

regression of tumour size might spare enough cancer stem cells to allow regrowth of the tumours. Therapies that are more specifically directed against cancer stem cells might result in much more durable responses and even cures of metastatic tumours (Figure 1).

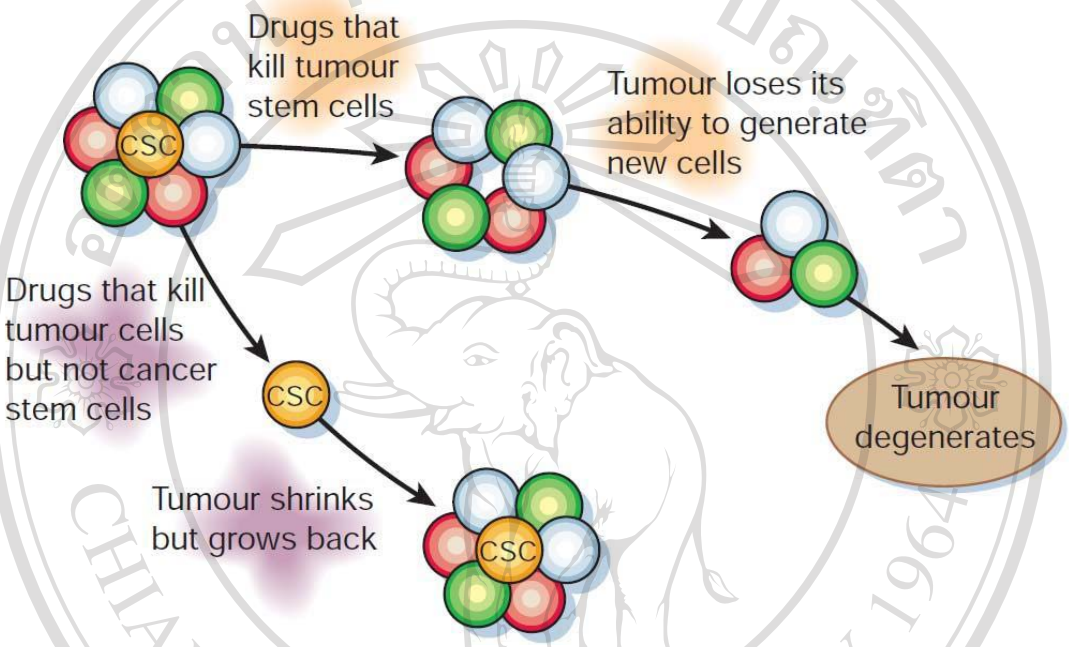


Figure 1. The scheme of conventional cancer therapy and therapy targeted against cancer stem cells (5).

2. Biochemistry and physiology of normal and cancer cells

Advancing knowledge of the cellular and molecular biology of processes that regulate cell proliferation, cell differentiation and cellular response to external signals has provided a number of potential targets for new approaches to treat cancer. This knowledge has provided a wealth of information about the biochemistry and biology of the cancer cell and about how a cancer cell differs from the normal cell in tissues, in which cancer arises. It is these difference that must be exploited in the development of the next generation of anti-cancer agents.

The key physicochemical properties which are tightly controlled are altered in normal cells causing changes in cellular biochemistry and physiology including cellular energetic state, buffering system, oxidative stress situation.

2.1. Energetic state

The cellular energetic status is crucial for the activity and viability of cells. This cellular bioenergetic state can be directly monitored by the cellular ATP content and the membrane potential including the mitochondrial membrane potential and proton gradient. Mitochondrial transmembrane potential ($\Delta\Psi m$), the driving force of cellular adenosine triphosphate (ATP) formation, is an important determinant of the energetic status and physiologic activity of the cell and also constitutes an obligatory step in cell-death programs (26-29). It is well documented that there are many cellular biochemical and physiological changes during carcinogenesis, particularly micro-environments of the mitochondria (30, 31). Our previous study reported on a non-invasive spectrofluorometric and flow cytometric technique for measuring the mitochondrial membrane potential ($\Delta\Psi m$) and proton gradient of erythomyeolgenous leakemic drug-sensitive (K562) and -resistant (K562/Adr) cells (14, 29). These studies demonstrated that the two parameters can be considered as sensitive indicators for studying the bioenergetic state of cancer cells that allow distinguishing the drug-sensitive from drug-resistant cells. In order to get more insight in leukemic cell biology, it seems important to compare the bioenergetic state of these cancer cells with those of normal leucocytes.

As tumor cells proliferate into the lumen, diffusion-reaction kinetics enforced by this separation result in hypoxia and acidosis in regions of the tumor the most distant from the basement membrane. This produces new evolutionary selection forces that promote constitutive up-regulation of glycolysis and resistance to acid-induced

toxicity (32). We hypothesize that the V-type H^+-ATPase of these cancer cells should be dysregulated which caused an elevate intraluminal pH of the lysosome (pH_v); these phenotypic adaptations are critical late steps in carcinogenesis conferring proliferative advantages even in normoxic conditions. As a consequence, cancer cells have a higher sensitivity to toxic agents than its corresponding normal parental cells. Once cancer cells become multidrug resistance phenotype, the V-type H^+-ATPase might be up-regulated resulting in a decrease in pH_v value thus an increase in the cellular digestion and exocytosis of xenobiotics.

2.2. Buffering system

An important intracellular and extracellular buffer is the CO_2/HCO_3^- system. Buffering occurs via the reversible protonation of bicarbonate anions leading to the release of CO_2,

$$H^+ + HCO_3^- \Leftrightarrow H_2CO_3 \Leftrightarrow CO_2 + H_2O.$$

Carbonic buffering is powerful provided (i) the surface membrane is highly permeable to CO_2 so that fluctuations of intracellular CO_2 can be damped via membrane permeation and (ii) the partial pressure of extracellular CO_2 is maintained relatively constant. As both CO_2 and HCO_3^- are low molecular weight solutes they will have high intracellular mobility (33). Carbonic buffer may therefore be expected to shuttle protons spatially within the cell, as illustrated in Figure 2. The ability of carbonic buffer to shuttle protons inside cells depends on the availability of the open, membrane-permeant form of the buffer (CO_2) from the extracellular medium, and on the speed of hydration of CO_2 to carbonic acid and back. The former is not a limiting factor, because membranes are highly permeable to CO_2, whereas the latter factor is slow.

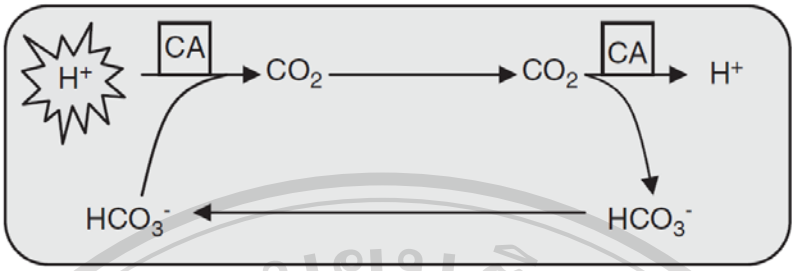


Figure 2. Carbonic buffer plays a role in providing additional mobile buffer for facilitating intracellular proton flux (34).

Experimental measurements of H_i^+ mobility have indicated that the presence of 5% CO_2 23 mM HCO_3^- enhances H_i^+ mobility by 2–5-fold (34-36) consistent with proton movement via the carbonic shuttle. This level of enhancement, however, requires the activity of the enzyme, carbonic anhydrase (CA), which catalyses the reversible hydration of CO_2. Inhibition of CA with acetazolamide attenuates the enhancement, indicating that the rate of proton shuttling is likely to be limited by the chemical hydration of CO_2 (35, 36). The regulation of H_i^+-mobility by CA is physiologically important, as an increase in apparent diffusion coefficient (D_H^{app}) will help to minimise the development of pH_i gradients, thus maintaining a spatially homogeneous pH_i. The relatively slow hydration of CO_2, even in the presence of active CA means, however, that the carbonic shuttle can minimise spatial pH_i gradients only when they are generated by a low rate of local acid production within the cell.

2.3. Oxidative stress

Free radicals are important intermediates constantly produced *in vivo* through a variety of normal metabolic processes as well as being common intermediates generated after exposure to drugs, xenobiotics or ionizing radiation (37). The last decade has seen a large interest in free radicals in biology and the pathogenesis of many diseases has been

associated with reactive oxygen species (ROS) (38). Furthermore, the uncontrolled generation of ROS may lead to aging, inflammation and neurodegenerative disorders (39). Although free radicals are usually considered to cause oxidative damage to the living organism, some of these such as nitric oxide ($^{\bullet}NO$) and the superoxide radical ($O_2^{\bullet-}$) also have beneficial functions including, for example, the regulation of blood pressure and blood flow (40), the induction of macrophage tumor cytotoxicity (41) and the phagocytic killing of harmful bacteria (42).

Under normal physiological conditions, it is estimated that up to 1% of the mitochondrial electron flow leads to the formation of $O_2^{\bullet-}$, the primary oxygen free radical produced by mitochondria. Interference with electron transport can dramatically increase $O_2^{\bullet-}$ production. While these partially reduced oxygen species can attack iron sulfur centers in a variety of enzymes, $O_2^{\bullet-}$ is rapidly converted within the cell to hydrogen peroxide (H_2O_2) by the superoxide dismutases (SOD1, SOD2 and SOD3). However, H_2O_2 can react with reduced transition metals, via the Fenton reaction, to produce the highly reactive hydroxyl radical ($^{\bullet}OH$), a far more damaging molecule to the cell. In addition to forming H_2O_2, $O_2^{\bullet-}$ radicals can rapidly react with nitric oxide (NO) to generate cytotoxic peroxynitrite anions ($ONOO^-$). Peroxynitrite can react with carbon dioxide, leading to protein damage via the formation of nitrotyrosine and lipid oxidation. The generation of ROS in normal cells is under tight homeostatic control. To eliminate ROS, biological anti-oxidants, including glutathione, α-tocopherol (vitamin E), carotenoids and ascorbic acid, will react with most oxidants. In addition, the anti-oxidant enzymes catalase and glutathione peroxidase eliminate H_2O_2 by converting it to O_2 and H_2O. However, when ROS levels exceed the anti-oxidant capacity of a cell, a deleterious condition known as oxidative stress occurs. Unchecked, excessive ROS can lead to the

destruction of cellular components, including lipids, protein DNA and ultimately cell death via apoptosis or necrosis (43).

Oxidative stress has been shown to up-regulate growth factors, including EGF and VEGF (44). Oxidative stress stimulation of growth factor signaling could also be ligand-independent. For example, H_2O_2 treatment of cell lines or cultured T cells has been shown to quickly induce tyrosine phosphorylation of multiple proteins including EGFR, PDGFR and the T cell receptor complex (45, 46). The transactivation of these receptors occurs in the absence of ligand and, in the case of EGF receptor, does not result in the endocytosis of the receptor; thus receptor signaling is prolonged (47). Such enhanced signaling has been demonstrated to stimulate an increase in cellular proliferation and transformation. The transactivation of growth factor receptors appears to trigger signal through several pathways, including the extracellular signal-regulated kinases (ERKs; a subset of the MAPKs), PI3K/AKT and phospholipase C-γ1 (46). The activation of each of these signaling pathways has been shown to induce either apoptosis or cell survival depending on the cell type and oxidative insult. Several other enzymatic pathways are modulated either directly or indirectly by oxidative stress. These include the stress-activated protein kinases JNK and p38, JAK/STAT, PKC and *ataxia telangiectasia* mutated, which also participate in signals leading to either cell survival or cell death (46). There is significant cross-talk between these signals, which complicates the task of establishing pathways of oxidative stress–induced apoptosis and presumably also oxidative stress–induced cell cycle re-entry (46).

ROS can also activate the transcription factor and tumor suppressor protein p53 (48). Although this protein plays an important role as a sensor of genotoxic stress and regulator of genes necessary for growth arrest and cell death, recent evidence suggests

that p53 activation can in turn activate genes, including anti-oxidants and heparin-binding EGF-like factor, that function in compensatory survival pathways (49). The p53 can also activate ERK and AKT, leading to an induction of COX-2, an important mediator of various proliferative diseases, including cancer (49). ROS and/or oxidative damage can activate gene transcription. However, as discussed previously, transcribed genes may be implicated in either cell survival or cell death. For example, increased NF-κB activity has been observed in the brains of Alziemer disease patients, in both neurons and astrocytes. Although the direct activation of NF-κB by ROS is controversial (50), the activities of this transcription factor can lend itself to pro- and antiapoptotic roles in the cell. In neurons, the activation of NF-κB has been linked to the activation of growth factors and COX-2 as well as anti-oxidants, specifically SOD2. In contrast, NF-κB activation in astrocytes and microglia results in the activation of pro-oxidants, including nitric oxide, in addition to growth factors (51).

Malignant cells in general are more active than normal cells in the production of O_2^- and are under intrinsic oxidative stress thus more vulnerable to damage by ROS-generating agents. The intrinsic oxidative stress in cancer cells was associated with the up-regulation of SOD and catalase protein expression, likely as a mechanism to tolerate increased ROS stress. The increase in SOD and catalase expression was observed both in primary human leukemia cells and ovarian cancer cells. Both malignant cell types were more sensitive to 2-methoxyestradiol (2-ME) than their normal counterparts, as demonstrated by the significant accumulation of O_2^- and subsequent apoptosis. The administration of ROS scavengers in combination with 2-ME prevented the accumulation of O_2^- and abrogated apoptosis induction (52).

3. Classification of polyphenols

In general, polyphenols or phenolic compounds stand for encompassed molecules that possess an aromatic ring bearing one or more hydroxyl substituents. Polyphenols are a class of phytochemicals found in high concentrations in wide varieties of higher plants and their derived-products, such as wine and tea. Natural polyphenols can range from simple molecules, such as phenolic acid, flavonoids and large highly polymerized compounds such as tannins. Conjugated forms of polyphenols are the most common, where various sugar molecules, organic acids and lipids are linked with the phenolic ring structure (53).

The polyphenols can be classified into the following categories:

1. Phenolic acids are simple molecules including the widely distributed hydroxybenzoic such as p-hydroxybenzoic, gallic acid, ellagic acid and hydroxycinnamic acids such as p-coumaric, caffeic acid, ferulic acid as indicated in Table 1 (54).

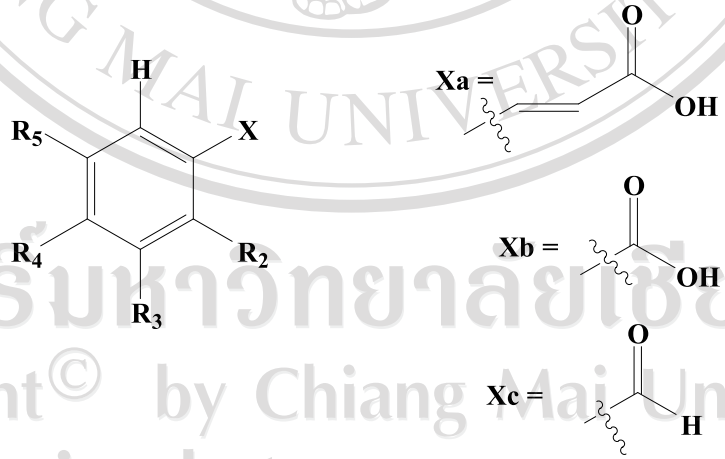


Table 1. Chemical structures of phenolic acids (54).

R2	R3	R4	R5	X	Name
H	H	H	H	A	cinnamic acid
OH	H	H	H	A	*o*-coumaric acid
H	H	OH	H	A	*p*- coumaric acid

Table 1. Chemical structures of phenolic acids (continued).

R2	R3	R4	R5	X	Name
H	OH	H	H	A	*m*- coumaric acid
H	OMe	OH	H	A	ferulic acid
H	OMe	OH	OMe	A	sinapic acid
H	OH	OH	H	A	caffeic acid
H	H	H	H	B	benzoic acid
OH	H	H	H	B	salicylic acid
H	H	OH	H	B	*p*-hydroxybenzoic acid
H	OMe	OH	H	B	vanillic acid
H	OMe	OH	OMe	B	syringic acid
H	OH	OH	H	B	protocatechuic acid
OH	H	H	OH	B	gentisic acid
OH	OH	OH	OH	B	gallic acid
H	OMe	OMe	H	B	veratric acid
H	OMe	OH	OMe	C	syringealdehyde
H	OMe	OH	H	C	vanillin

2. Flavonoids are a group of chemical compounds, low molecular weight phenylbenzopyrones as indicated in Table 2 (55). They are usually subdivided into 7 subgroups:

1. Flavonols: quercetin, kaempferol and myricetin
2. Flavones: luteolin and apigenin
3. Flavanones: hesperetin, naringenin and eriodictyol
4. Flavanols: (+)-catechin, (+)-gallocatechin, (-)-epicatechin, (-)-epigallocatechin, (-)-epicatechin 3-gallate, (-)-epigallocatechin 3-gallate
5. Flavanonol: taxifolin
6. Isoflavone: daidzein, genistein
7. Anthocyanidins: cyanidin, delphinidin, malvidin, pelargonidin, peonidin, petunidin

Table 2. Chemical structure of flavonoids.

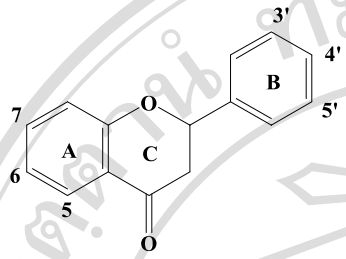
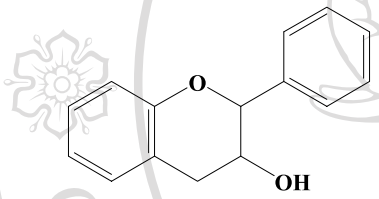
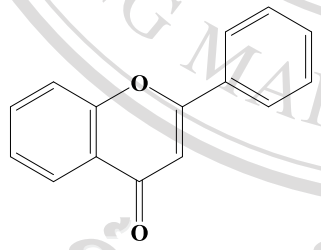
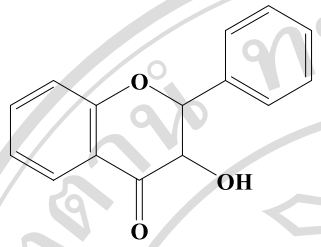
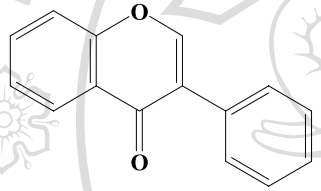
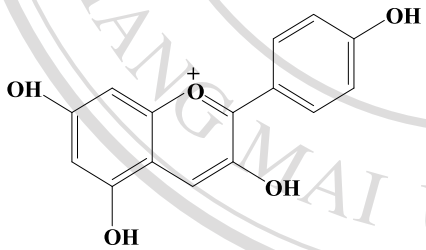
Structure Formula	Representative Flavonoids	Substituents					
		5	6	7	3’	4’	5’
 **Flavanon**	Erioditol	OH	H	OH	OH	OH	H
	Hesperitin	OH	H	OH	OH	OMe	H
	Nariginin	OH	H	OH	H	OH	H
 **Flavanol**	Catechin	H	H	H	H	H	H
	Gallocatechin	H	H	H	OH	OH	OH
 **Flavone**	Apigenin	OH	H	OH	H	OH	H
	Chrysin	OH	H	OH	H	H	H
	Luteolin	OH	H	OH	OH	OH	H
	Myricetin	OH	H	OH	OH	OH	OH
	Quercetin	OH	H	OH	OH	OH	H

Table 2. Chemical structure of flavonoids (continued).

Structure Formula	Representative Flavonoids	Substituents					
		5	6	7	3’	4’	5’
 **Flavanonol**	Taxifolin	OH	H	OH	OH	OH	H
 **Isoflavone**	Daidzein	H	H	OH	H	OH	H
	Genistein	OH	H	OH	H	OH	H
	Glycitein	OH	OMe	OH	H	OH	H
	Formononetin	H	H	OH	H	OMe	H
 **Anthocyanidin**	Delphinidin	OH	H	OH	OH	OH	OH
	Cyanidin	OH	H	OH	OH	OH	H
	Malvidin	OH	H	OH	OMe	OH	OMe
	Peonidin	OH	H	OH	OMe	OH	H
	Pelargonidin	OH	H	OH	H	OH	H
	Petunidin	OH	H	OH	OH	OH	OMe

3. Tannins are large molecules including condensed tannins (proantho-cyanidins), derivative tannins and hydrolysable tannins as indicated in Figure 3 (56).

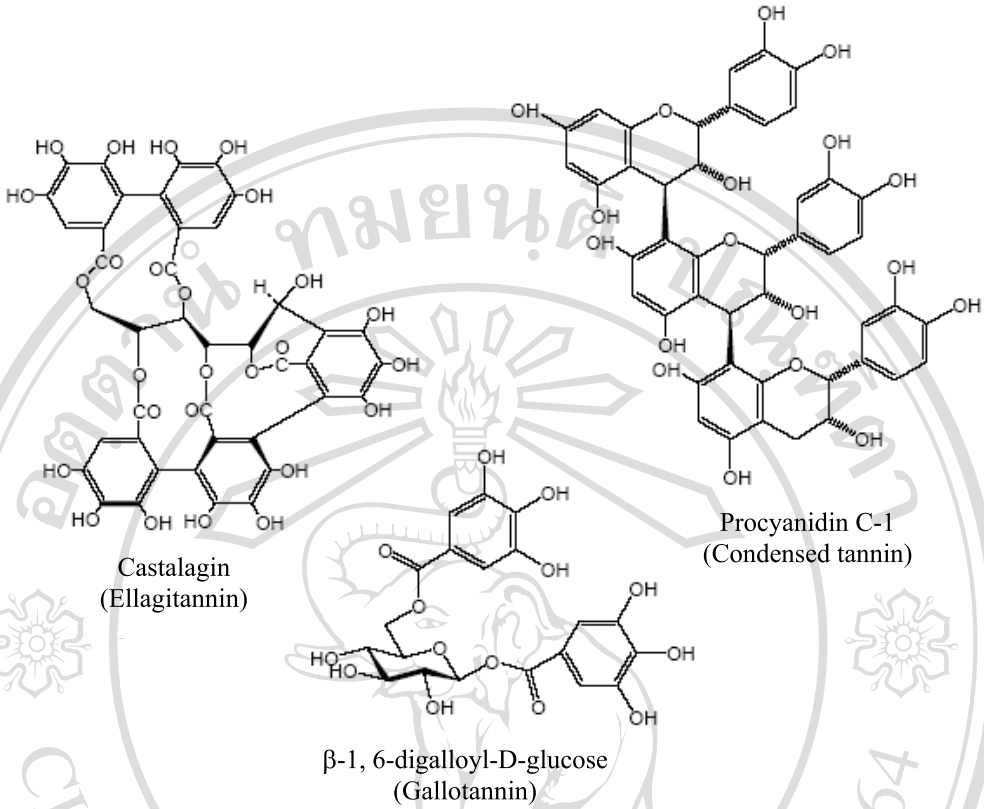


Figure 3. Chemical structure of tannins (56).

4. Anti-oxidant activities of polyphenols

Several polyphenols have clearly shown anti-oxidant properties *in vitro* as chain breakers or radical scavengers depending on their chemical structures, which also affect their anti-oxidant power. A hierarchy has been established for the different polyphenolic compounds within each class on the basis of their capability to protect lipids, proteins or DNA against oxidative injury. Flavonoids efficiently scavenge and chelate the radical species, which can undergo cellular oxidative stress or may serve as an intracellular electron donor for a transplasma membrane oxido-reductase, suggesting that the flavonoids exert beneficial effects under oxidative stress conditions (57-61). Consequently, many of their biological actions have been attributed to those anti-oxidant

properties (62, 63). Moreover, the tea polyphenol epigallocatechin gallate (EGCG) has shown an anti-oxidant activity by reducing reactive oxygen species levels *in vitro* (64). These properties, however, appears now to be a simplistic way to conceive for their activity. First, pro-oxidant effects of polyphenols have also been described to have opposite effects on basic cell physiological processes (65). For example, if as anti-oxidants they improve cell survival, as pro-oxidants they may indeed induce apoptosis, cell death and block cell proliferation (66). Moreover, intracellular redox status, which is influenced by anti-oxidants, can regulate different factors, e.g., NFκB, which in turn regulates various cell activities (67, 68).

Various health benefits of polyphenolic anti-oxidants have been reported, such as anti-aging consequences and prevention of cancer, heart and peripheral artery disease (69, 70). Oxidation of DNA is likely to be an important cause of mutations that potentially can be reduced by dietary anti-oxidants. Among bioflavonoids, quercetin is frequently used for testing the pharmacological properties. The potential beneficial use of quercetin in preventing ischemia/reperfusion-induced myocardial damage by reactive oxygen species has been reported. By using a normal cell, such as the H9c2 cardiomyoblast cell, quercetin could protect H9c2 cells from hydrogen peroxide-induced apoptosis (71). It was also reported that quercetin showed a higher value of anti-oxidant activity than Vitamin C, Vitamin E and β-carotene on a molar basis (72) and probably due to the anti-oxidant action, it prevented the generation of reactive oxygen species by cyclosporine and thereby suppressed the cyclosporine-induced nephrotoxicity in rats (73). The latter suggested that quercetin is safe and has potentially protective action against cyclosporine toxicity *in vivo*.

5. Effect of polyphenols on cellular biochemistry and physiology

5.1. Apoptosis-inducing activity against cancer cells

Apoptosis is of Greek origin, having the meaning "falling off or dropping off", in analogy to leaves falling off trees or petals dropping off flowers. This analogy emphasizes that the death of living matter is an integral and necessary part of the life cycle of organisms. The apoptotic mode of cell death is an active and defined process which plays an important role in the development of multicellular organisms and in the regulation and maintenance of the cell populations in tissues upon physiological and pathological conditions. Thus, dysfunction or dysregulation of the apoptotic program is implicated in a variety of pathological conditions. Defects in apoptosis can result in cancer, auto-immune diseases and spreading of viral infections, while neurodegenerative disorders, AIDS and ischaemic diseases are caused or enhanced by excessive apoptosis (74).

Apoptotic cells can be recognized by stereotypical morphological changes: the cell shrinks, shows deformation and looses contact with its neighboring cells. Its chromatin condenses and marginates at the nuclear membrane; the plasma membrane is blebbing or budding, and finally the cell is fragmented into compact membrane-enclosed structures, called “apoptotic bodies” which contain cytosol, the condensed chromatin, and organelles. The apoptotic bodies are engulfed by macrophages and thus are removed from the tissue without causing an inflammatory response (75). Apoptosis is in contrast to the necrotic mode of cell death, in which case the cells suffer a major insult, resulting in a loss of membrane integrity, swelling and disruption of the cells. During necrosis, the cellular contents are released uncontrolled into the cell's environment which results in

damage of surrounding cells and a strong inflammatory response in the corresponding tissue as shown in Figure 4 (76).

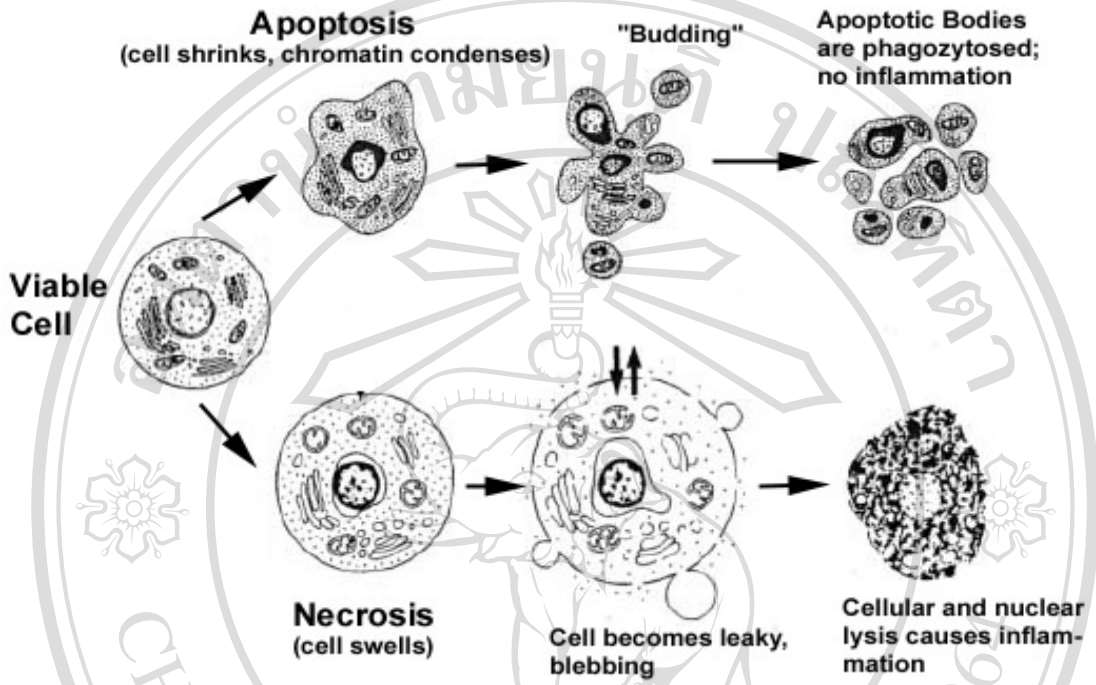


Figure 4. Hallmarks of the apoptotic and necrotic cell death process (http://www.celldeath.de/encyclo/aporev/aporev.htm).

The basic apoptotic signaling pathways and molecular machinery responsible for the induction and execution of apoptosis were elucidated. The most important signaling molecules and cellular structures will be discussed in context of their function and of the mechanisms in which they are involved in the initiation, mediation, execution, and regulation of apoptosis. The impression of the sophisticated interplay between factors that promote or suppress apoptosis resulting in a complicated regulatory network is depicted in Figure 5. Apoptosis can be induced in response to various signals from inside and outside the cell, e.g. by ligation of so-called death receptors or by cellular stress triggered by oncogenes, irradiation or drugs. Signals emanating from death receptors initially

activate the Death Inducing Signaling Complex (DISC) which mediates activation of the initiator caspase-8. Activated caspase-8 initiates a caspase cascade by processing the effector caspases-3, -6, and -7 which in turn cleave a number of protein substrates. Cleavage of caspase substrates eventually leads to the characteristic morphological and biochemical features of apoptosis. The initiator caspases are recruited to and activated at death-inducing signaling complexes either in response to the ligation of cell surface death receptors (extrinsic apoptosis pathways) or in response to signals originating from inside the cell (intrinsic apoptosis pathways). In some cell systems, this direct caspase cascade is sufficient to elicit apoptosis on its own, whereas in other cases the signal coming from the DISC must be amplified by the proteolytic activation of the BH3-only protein Bid by caspase-8 with subsequent induction of apoptotic events at the mitochondria (77-79).

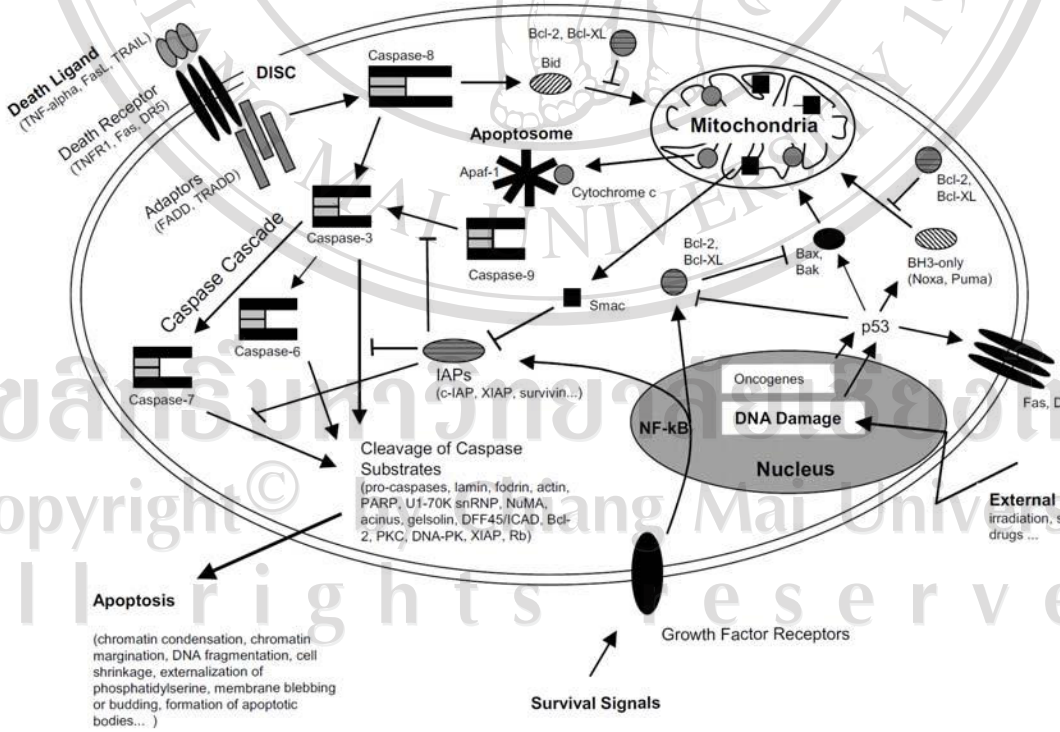


Figure 5. Schematic representation of some major apoptotic signalling pathways (http://www.celldeath.de/encyclo/aporev/aporev.htm).

Mitochondrial apoptotic signaling includes the release of cytochrome c from the mitochondrial inter-membrane space to the cytosol, where it contributes to the formation of the apoptosome which consists of cytochrome c, Apaf-1 and dATP. The apoptosome activates caspase-9, which is another initiator caspase and thus is able to mediate the caspase cascade by activating caspase-3. Another mitochondrial pro-apoptotic factor is Smac which acts by inhibiting the inhibitors of apoptosis proteins (IAPs) from blocking caspase activity. The IAPs are a family of proteins with anti-apoptotic activity by directly inhibiting caspases. IAP expression can be up-regulated in response to survival signals, such as those coming from growth factor receptors, e.g. by activation of the transcription factor, NFκB, thus providing a means to suppress apoptosis signaling. Of central importance are the anti-apoptotic Bcl-2 family members, such as Bcl-2 and Bcl-XL, which counteract the action of BH3-only proteins, such as Bid, but also of pro-apoptotic Bax and Bak, thus inhibiting mitochondrial pro-apoptotic events. Apoptotic signals coming from the inside of the cell frequently have their origin within the nucleus, being a consequence of DNA damage induced by irradiation, drugs or other sort of stress. DNA damage in most cases eventually results in the activation of the p53 transcription factor which promotes expression of proapoptotic Bcl-2 members and suppresses anti-apoptotic Bcl-2 and Bcl-XL. Other organelles besides mitochondria and the nucleus, such as the ER and lysosomes, also have been implicated in apoptotic signaling pathways, and it should be kept in mind that presumably hundreds of proteins are part of an extremely fine-tuned regulatory network consisting of pro- and anti-apoptotic factors (77, 80).

According to several studies, polyphenols are considered as effective inhibitors of cancer cell growth and induce apoptosis in various cancer cell lines (81-83). The

relationship of chemical structures of polyphenols and then anti-cancer properties has been widely studied with *in vitro* and *in vivo* system and polyphenol concentrations required for anti-cancer effects vary depending on the types of cancer cell lines (15). It was also reported that flavonoids induce apoptosis in cancer cells via decrease in $\Delta\Psi_m$, due to cytochrome c release and induction of caspase-9 processing (84, 85). In fact, quercetin provoked its cytotoxicity at the mitochondria level, impairing the mitochondrial energetic state followed by an induction of apoptosis and inhibition of cancer cell growth (15). In addition, quercetin induced apoptosis in the K562 human chronic myeloid leukemia, Molt-4 acute T-lymphocytic leukemia, Raji Burkitt lymphoma, and MACS mucinous cystoadenocarcinoma of ovary cell lines in a dose- and time-dependent manner (86). The later studies demonstrated that plant polyphenols can induce apoptosis through the mitochondrial pathway. For example, tannins, woodfordin I was able to suppress the proliferation and induce apoptosis in K562 cells through the loss of $\Delta\Psi_m$, transient generation of reactive oxygen species (ROS), transient elevation of intracellular Ca^{2+} concentration, cytosolic accumulation of cytochrome c and activation of caspase-9 and 3, but not caspase-8 (87). Zhao *et al*., showed that the most abundant green tea polyphenol, epigallocatechin-3-gallate (EGCG) elevated the activities of caspase-9, increased the release of cytochrome c from the mitochondria, and inhibited the protein expression of Bcl-2 leading to apoptosis in nasopharyngeal carcinoma HNE2 cells (88). Phenolic acids including caffeic and protocatechuic acid exhibited anti-proliferative and apoptotic action via the Fas/FasL system in T47D human breast cancer cells (89). Recent studies demonstrated that polyphenolic compounds revealed anti-proliferative and apoptosis-inducing activity against cancer cells through the activation of caspase-3 (90-93). Moreover, flavonoids including quercetin, luteolin and genistein inhibit cell proliferation

and induce apoptosis in various cancer cells via the blockade of different signalling pathways such as p38, ERK MAPK and PI3K/Akt (94-97).

5.2. Multidrug-resistant cancer cells

There are two general classes of resistance to anti-cancer drugs: those that impair delivery of anti-cancer drugs to tumor cells and those that arise in the cancer cell itself due to genetic and epigenetic alterations that affect drug sensitivity. Impaired drug delivery can result from poor absorption of orally administered drugs, increased drug metabolism or increased excretion, resulting in lower levels of drug in the blood and reduced diffusion of drugs from the blood into the tumor mass (98, 99). Recent studies have emphasized the importance of the tumor vasculature and an appropriate pressure gradient for adequate drug delivery to the tumor (100). In addition, some cancer cells that are sensitive to chemotherapy as monolayer cells in culture become resistant when transplanted into animal models (101). This indicates that environmental factors, such as the extracellular matrix or tumor geometry, might be involved in drug resistance. Cancer cells grown in culture as three-dimensional spheroids, mimicking their *in vivo* geometry, have also been shown to become resistant to cancer drugs (99, 101, 102). Much remains to be learned about this type of drug resistance and its role in clinical oncology. Cellular mechanisms of drug resistance have been intensively studied, as experimental models can be easily generated by *in vitro* selection with cytotoxic agents. Cancer cells in culture can become resistant to a single drug, or a class of drugs with a similar mechanism of action, by altering the drug's cellular target or by increasing repair of drug-induced damage, frequently to DNA. After selection for resistance to a single drug, cells might also show cross-resistance to other structurally and mechanistically unrelated drugs, a phenomenon that is known as 'multidrug resistance' (MDR) (103). This might explain why treatment regimens that combine multiple agents with different targets are not more effective.

As illustrated in Figure 6, different types of cellular multidrug resistance have been described. Resistance to natural-product hydrophobic drugs sometimes known as classical multidrug resistance generally is result from expression of ATP-dependent efflux pumps with broad drug specificity. These pumps belong to a family of ATP-binding cassette (ABC) transporters that share sequence and structural homology. So far, 48 human ABC genes have been identified and divided into seven distinct subfamilies (ABCA to ABCG) on the basis of their sequence homology and domain organization (104). The structures of ABC transporters can be grouped in three categories (Figure 7). Multidrug resistance 1 (MDR1) or P-glycoprotein (P-gp) and multidrug resistance-associated protein 4, 5, 7 (MRP4, 5, 7) have 12 transmembrane domains and two ATP-binding sites (Figure 7 a). The structures of MRP1, 2, 3 and 6 are similar in that they possess two ATP-binding regions. They also contain an additional membrane domain that is composed of five transmembrane segments at the amino-terminal end, giving them a total of 17 transmembrane domains (Figure 7 b). The 'half-transporter' ABCG2 contains six transmembrane domains and one ATP-binding region, in this case, on the amino-terminal side (N) of the transmembrane domain. In other 'half-transporters', such as the transporter associated with antigen processing (TAP), the ATP-binding cassette is found on the carboxy-terminal (C) side of the transmembrane domain. Half-transporters are thought to homodimerize or heterodimerize to function (Figure 7 c). Resistance results because increased drug efflux lowers intracellular drug concentrations. Drugs that are affected by classical multidrug resistance include the Vinca alkaloids (vinblastine and vincristine), the anthracyclines (doxorubicin and daunorubicin), the RNA transcription inhibitor actinomycin-D and the microtubule-stabilizing drug paclitaxel (105). Major ABC transporters associated with MDR, chemotherapy substrates and MDR inhibitors common other systematic substrates inhibitors are indicated in Table 3.

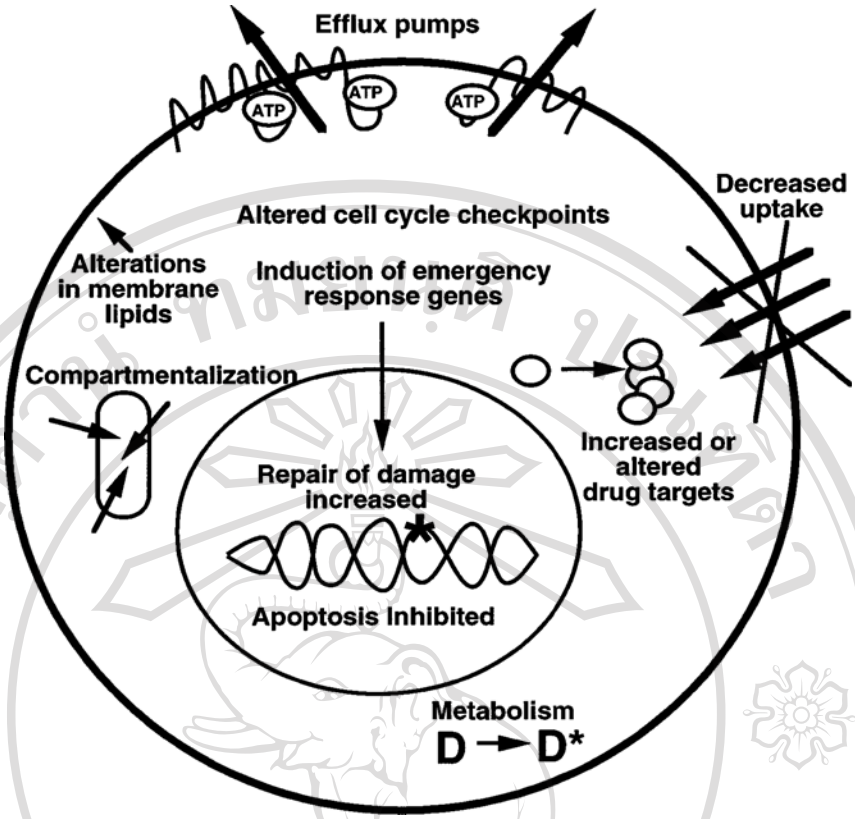


Figure 6. Cellular factors that cause drug resistance (106).

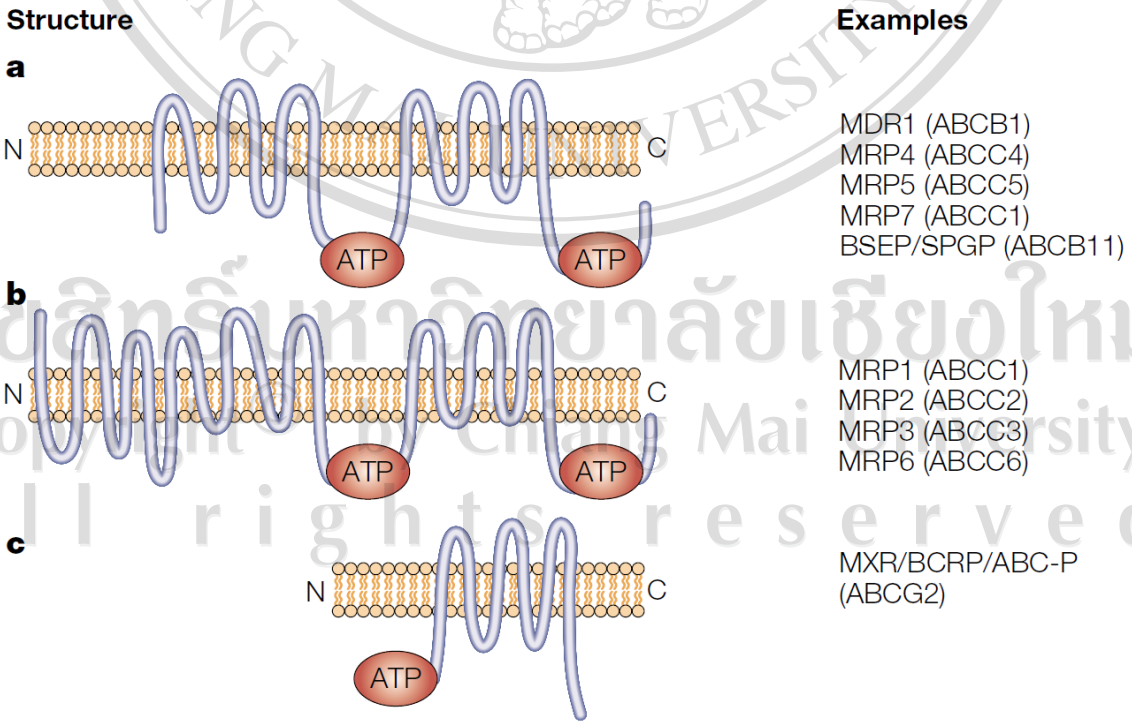


Figure 7. Structures of ABC transporters known to confer drug resistance (107).

Table 3. Major ABC transporters associated with MDR, chemotherapy substrates and MDR inhibitors common other systematic substrates inhibitors (108).

Common name	Other names	Systematic name	Substrates	Inhibitors
P-gp	MDR1	ABCB1	Adriamycin Actinomycin-D Bisantrene Daunorubicin Docetaxel Doxorubicin Etoposide Epirubicin Homoharringtonine Mitoxantrone Paclitaxel Teniposide Topotecan Vinblastine Vincristine Vinorelbine VP-16	Anthranilamide Cyclosporine D NSC-38721 (mitotane) Pipecolinate Quinoline OC-144-093 PSC-833 (valspodar) MS-209 LY-335979 (zosoquidar) XR-9576 (tariquidar) R-101933 (laniquidar) VX-710 (biricodar) GF-120918 (elacridar) ONT-093 Isothiocyanates Diallyl sulfide PK11195 Amooranin siRNA tRA 98006 Agosterol A Flavonoids
MRP1	–	ABCC1	Doxorubicin Daunorubicin Etoposide Epirubicin Methotrexate Paclitaxel Vincristine Vinorelbine	MS-209 XR-9576 (tariquidar) VX-710 (biricodar) Isothiocyanates tRA 98006 Agosterol A Rifampicin NSAIDs
MRP2	CMOAT	ABCC2	Cisplatin CPT-11 (irinotecan) Doxorubicin Etoposide Methotrexate Mitoxantrone Vincristine Vinblastine SN-38	XR-9576 (tariquidar) VX-710 (biricodar) Isothiocyanates tRA 98006
BCRP	MXR1, ABC-P	ABCG2	Bisantrene Camptothecin Daunorubicin Doxorubicin Epirubicin Flavopiridol Mitoxantrone SN-38 Topotecan CPT-11 (irinotecan)	GF-120918 (elacridar) tRA 98006 Flavonoids Phytoestrogens Imatinib mesylate Fumitremorgin C TAG- 139

Resistance can also be mediated by reduced drug uptake. Water-soluble drugs that 'piggyback' on transporters and carriers that are used to bring nutrients into the cell, or agents that enter by means of endocytosis, might fail to accumulate without evidence of increased efflux. Examples include the antifolate methotrexate, nucleotide analogues,

such as 5-fluoro-uracil and 8-azaguanine, and cisplatin (109, 110). MDR can also result from activation of coordinately regulated detoxifying systems, such as DNA repair and the cytochrome P450 mixed function oxidases. Indeed, coordinate induction of the multidrug transporter P-glycoprotein (P-gp) and cytochrome P450 3A has been observed (111). This type of multidrug resistance can be induced after exposure to any drug. Recent evidence indicates that certain orphan nuclear receptors, such as SXR, might be involved in mediating this global response to environmental stress (112).

Finally, resistance can result from defective apoptotic pathways. This might occur as a result of malignant transformation; for example, in cancers with mutant or non-functional p53 protein (113). Alternatively, cells might acquire changes in apoptotic pathways during exposure to chemotherapy, such as alteration of ceramide levels (114) or changes in cell cycle machinery, which activate checkpoints and prevent initiation of apoptosis. An important principle in multidrug resistance is that cancer cells are genetically heterogeneous. Although the process that results in uncontrolled cell growth in cancer favours clonal expansion, tumor cells that are exposed to chemotherapeutic agents will be selected for their ability to survive and grow in the presence of cytotoxic drugs. These cancer cells are likely to be genetically heterogeneous because of the mutator phenotype. So, in any population of cancer cells that is exposed to chemotherapy, more than one mechanism of multidrug resistance can be present. This phenomenon has been called “multifactorial multidrug resistance”.

The MDR phenomenon is a major cause of treatment failure in cancer therapy and associated with a decreased intracellular accumulation of anti-cancer drugs, consequently there is a decline in the therapeutic effect due to an overexpression of MDR transporter proteins including P-glycoprotein (P-gp or MDR1), multidrug resistance-associated protein (MRP), and lung resistance-related protein (LRP), breast cancer

resistant protein (BCRP) (106, 107, 115, 116). The P-gp and MRP-1 extrude chemotherapeutic drugs by using ATP hydrolysis as a source of energy (117, 118). In addition, several studies suggested that a modification of cellular anti-cancer drug distribution might be perturbed by an altered pH gradient across different cell compartments. Particularly, acidic organelles such as lysosomal sequestration following enhanced exocytosis, which favors a reduced intracellular accumulation of anti-cancer drugs, reducing efficiency, was characterized in various MDR cell types (119, 120). However, the combination of P-gp-mediated flux and the intracellular drug sequestration governed by the MDR phenotype, was not considered by those researchers.

Experimental approaches aiming to determine the direct interaction between flavonoids and P-gp were studied by many research groups. On one hand, the fixation of flavonoids at vicinal ATP-binding site was investigated by measuring the resonance-energy transfer of the tryptophan-intrinsic fluorescence of H6-NBD2, a highly soluble recombinant protein, from mouse P-gp and flavonoids (121). Similar results were obtained from the series of experiments dealing with the inhibition of photolabeling of ATP analogues on the ATP-binding site within the C-terminal nucleotide-binding domain of mouse P-gp. De Wet and coworkers showed the structure-activity relationships of 30 flavonoids on their ability to bind the vicinal ATP- and steroid-binding site (122). On the other hand, It was reported that flavonols (quercetin, kaempferol and galangin) were potent stimulators of the P-gp-mediated efflux of 7,12-dimethylbenz(a)anthracence in multidrug-resistant breast cancer cells (123). Consistently with previously cited data, it was found that galangin, kaempferol and quercetin reduced [^{14}C] ADR accumulation and this phenomenon was blocked by verapamil, vinblastine, and quinidine in HCT-15 colon cells (124).

Several studies suggested that MDR cells need more cellular ATP than that of their corresponding sensitive cells (125-128). A strong evidence is that ATP depletion in MDR cells, even partial, will block P-glycoprotein and MRP1 pump activity leading to an increase in cellular drug accumulation (129, 130). Indeed, understanding of the source of cellular ATP production and the cellular energetic state of MDR cells is crucial data to overcome MDR phenomena. In other words, we must get insight into the important role that mitochondria play in the MDR phenotype since they supply the cellular ATP pool. The $\Delta\Psi_m$ is a sensitive indicator which indicates the energetic state of mitochondria and cells. An increase followed by a decrease in mitochondrial membrane potential ($\Delta\Psi_m$) value was associated with an induction of apoptosis. In these respects, the disruption of the $\Delta\Psi_m$ can be considered as a useful strategy to overcome MDR phenomenon.

Besides amplifying and mediating extrinsic apoptotic pathways, mitochondria also play a central role in the integration and propagation of death signals originating from inside the cell such as DNA damage, oxidative stress, starvation that are also induced by chemotherapeutic drugs (131, 132). It can be noted that an increasing concentration of quercetin causes the $|\Delta\Psi_m|$ decrease in very narrow range (from 160 ± 1.0 mV to 150 ± 0.6 mV for K562 and from 145 ± 1.2 mV to 135 ± 1.1 mV in K562/Adr), whereas the percentage of early apoptotic cells increases in a greater degree of range (from 1.5 ± 0.4% to 45 ± 3.2%) (15). Most apoptosis-inducing conditions involve the disruption of $\Delta\Psi_m$ leading to the socalled permeability transition (PT), a sudden increase of the inner mitochondrial membrane permeability. Concomitantly, osmotic mitochondrial swelling has been observed by influx of water into the matrix with eventual rupture of the outer mitochondrial membrane, resulting in the release of pro-apoptotic proteins from the mitochondrial inter-membrane space into the cytoplasm (133,

134). Released proteins include cytochrome c, which activates the apoptosome and therefore the caspase cascade, but also other factors such as the apoptosis-inducing factor (AIF), endonuclease endo-G, Smac/Diablo and Htr/Omi (135-138). Interestingly, PT is always followed by a decrease in $\Delta\Psi_m$, but loss of $\Delta\Psi_m$ is not always caused by PT, and cytochrome c release has been observed even in absence of $\Delta\Psi_m$ (133, 139). Several possible mechanisms for PT have been proposed, but there appears to exist consent that a so-called permeability transition pore (PTP) is formed consisting of the adenin nucleotide translocator (ANT) and the voltage-dependent anion channel (VDAC) as its core components. ANT is the most abundant protein of the inner mitochondrial membrane and as a transmembrane channel is responsible for the export of ATP in exchange with ADP (140).

5.3. NFκB-regulated gene expression in cancer cells

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 (141, 142). In mammalian cells, the NFκB/Rel family consists of five members: RelA (p65), RelB, c-Rel, p50/p105 (NFκB1), and p52/p100 (NFκB2). All proteins of the NFκB family share a Rel homology domain in their N-terminus. There are two structural classes of NFκB proteins including class I (p50/p105 and p52/p100) and class II (RelA, RelB and c-Rel). Both classes of proteins contain an N-terminal DNA-binding domain (DBD), which also serve as a dimerization interface to other NFκB transcription factors and in addition binds to the inhibitory IκBα protein. The C-terminus of class I proteins contains a number of ankyrin repeats and has a trans-repression domain (TRD). In contrast, the C-terminus of class II proteins has a trans-activation domain (TAD) (Figure 8). The NFκB1 and NFκB2 proteins are synthesized as large precursors,

p105 and p100, which undergo processing to generate the mature NFκB subunits, p50 and p52, respectively. The processing of p105 and p100 is mediated by the ubiquitin/proteasome pathway and involves selective degradation of their C-terminal region containing ankyrin repeats. Whereas the generation of p52 from p100 is a tightly regulated process, p50 is produced from constitutive processing of p105 (143, 144). In most cells, NFκB is composed of a heterodimer of p65 and p50 where the p65 protein is responsible for the trans-activation 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 (145), 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, its translocation into the nucleus and induction of gene transcription. Although there is a broadening complexity to NFκB signaling, the two most recognized pathways are the so-called “canonical” and “non-canonical” (Figure 9). The former depends on NEMO, IKKβ activation, nuclear localization of RelA/p50 dimers, and is associated with inflammation, while the latter depends on IKKa activation probably via the upstream kinase NIK, nuclear localization of p52/ RelB dimers, and is important in lymphoid organogenesis (146). Both pathways of NFκB activation have now been implicated in carcinogenesis (147, 148).

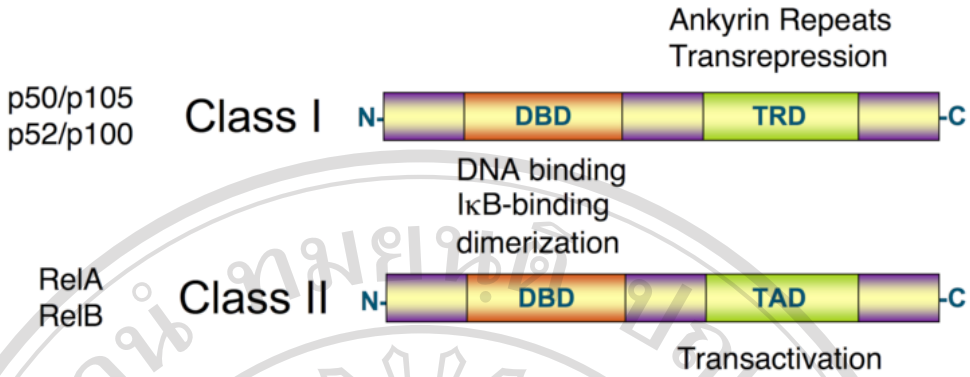


Figure 8. Schematic diagram of NFκB/Rel protein structure (http://en.wikipedia.org/wiki/File:NFκB_structure_schematic.png).

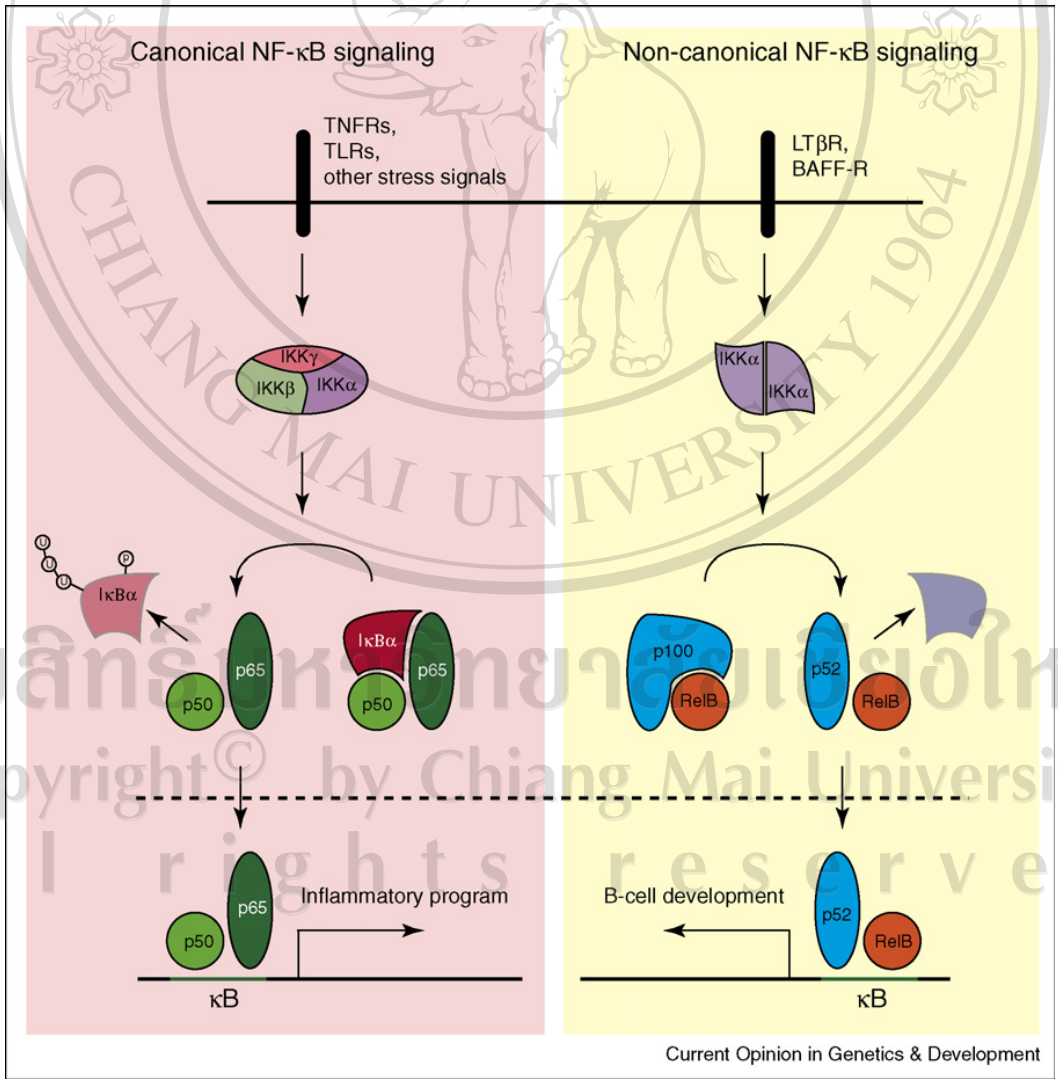


Figure 9. NFκB signaling pathways (Willscott *et al*., 2008).

NFκB has a role in oncogenesis and regulation of cancer therapeutic sensitivity. Overexpression, amplification, and rearrangements of different genes related to NFκB have been observed in tumors (149). NFκB is activated in response to various inflammatory stimuli including cytokines, mitogens, bacterial products, viral proteins, and apoptosis-inducing agents (150, 151). 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* (152). NFκB, possibly through the activation of the anti-apoptotic genes, plays a key role in the protection of cells against inducers of apoptosis including chemotherapeutic drugs (153). Several mechanisms including increased expression of NFκB proteins, mutations and/or deletions in IκBα gene, and increased IκBα turnover, are involved in NFκB hyperactivation in tumor cells (149, 154). As such, various therapeutic strategies aim to decrease chronic hyperactivated NFκB by pharmacological as well as phytomedicinal approaches in cancer (155-159). NFκB-regulated genes are involved in cell death, invasiveness, proliferation, angiogenesis, inflammation and multidrug resistance.

Cancer cells contain multiple signal transduction pathways of which the activities 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 this observation, a greater emphasis has begun to be placed on multifocal natural compounds such as polyphenols, withanolides, xanthones, indanones, curcuminoids which simultaneously inhibit multiple inter-linked signal transduction/survival pathways (156, 160-164). 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 (165). As such, over the past decades, researchers searching for new drugs to use in oncology have refocused on natural products (165, 166).

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 (162). Vanden Berghe *et al*., showed that phyto-estrogenic soy isoflavones can selectively block nuclear NFκB transactivation of specific NFκB target genes independently of their estrogenic activity in highly metastatic breast cancer cells (158). 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 (AP-1) and NFκB] and inflammatory protein [cyclo-oxygenase-2 (Cox-2)] (167, 168). The NFκB and Akt kinase pathway, which play critical roles in inflammation, vascular homeostasis and angiogenesis, were repressed by polyphenolic compound, deguelin in human vascular endothelial cells and *HT1080*

fibrosarcoma cells and chronic lymphocytic leukemia cells (169-171). 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 (172). The latter treatment blocks nuclear translocation of the transcription factor nuclear factor kappaB 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 compounds exhibit anti-inflammatory activity in activated macrophages by inhibiting NFκB signaling pathway (173-175). Dijsselbloem and coworkers demonstrated that genistein inhibits IL-6 gene expression by modulating the transcription factor NFκB in TLR4-stimulated dendritic cells (176). Pycnogenol inhibits tumor necrosis factor-alpha-induced NFκB activation and adhesion molecule expression in human vascular endothelial cells (177). 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 (178). 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 (179).