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COMPREHENSIVE INVITED REVIEW

Flavonoids as Protein Kinase Inhibitors for Cancer Chemoprevention: Direct Binding and Molecular Modeling

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Abstract

Protein kinases play crucial roles in the regulation of multiple cell signaling pathways and cellular functions. Deregulation of protein kinase function has been implicated in carcinogenesis. The inhibition of protein kinases has emerged as an important target for cancer chemoprevention and therapy. Accumulated data revealed that flavonoids exert chemopreventive effects through acting at protein kinase signaling pathways, more than as conventional hydrogen-donating antioxidants. Recent studies show that flavonoids can bind directly to some protein kinases, including Akt/protein kinase B (Akt/PKB), Fyn, Janus kinase 1 (JAK1), mitogen-activated protein kinase kinase 1 (MEK1), phosphoinositide 3-kinase (PI3K), mitogen-activated protein (MAP) kinase kinase 4 (MKK4), Raf1, and ζ chain-associated 70-kDa protein (ZAP-70) kinase, and then alter their phosphorylation state to regulate multiple cell signaling pathways in carcinogenesis processes. In this review, we report recent results on the interactions of flavonoids and protein kinases, especially their direct binding and molecular modeling. The data suggest that flavonoids act as protein kinase inhibitors for cancer chemoprevention that were thought previously as conventional hydrogen-donating antioxidant. Moreover, the molecular modeling data show some hints for creating natural compound-based protein kinase inhibitors for cancer chemoprevention and therapy. *Antioxid. Redox Signal.* 13, 691–719.

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I. Introduction

Protein kinase is a type of enzyme that transfers phosphate groups from high-energy donor molecules such as adenosine triphosphate (ATP) to specific substrates. This process is referred to as phosphorylation. According to the catalytic specificity, protein kinases can be subdivided into three categories: tyrosine (Tyr) kinase, serine/threonine (Ser/Thr) kinase, and kinase specific for both Tyr and Ser/Thr. Phosphorylation of these sites in target proteins leads to the activation of signal transduction pathways, which play important roles in a great number of biological processes, including cell growth, differentiation, development, and death (35, 235). The activities of protein kinases are stringently regulated in homeostasis systems. However, protein kinases can be deregulated under pathological conditions, leading to perturbation of protein kinase-mediated cell signaling pathways and resulting in various disorders, including cancer, diabetes, and inflammation (184). In particular, many protein kinases are intimately involved in the processes leading to tumor cell proliferation and survival (86, 117, 210). These protein kinases have transforming capacity and are therefore considered to be oncogenic. The constitutive activity of this class activity of such protein kinases makes them essential for survival and/or proliferation of the cancer cell. Thus, regulation of protein kinases has been shown to be a promising strategy for cancer chemoprevention and therapy (42, 59, 200, 235). Moreover, data from the Human Genome Project has revealed that 518 protein kinases are encoded in the human genome (18, 129, 207), and each cell will have approximately 50–100 protein kinases, thereby creating an enormous repertoire of potential targets for drug discovery (13, 94, 119, 191). Alterations in multiple cellular signaling pathways are frequently found in many cancer cells. It may be the reason why the specific inhibitors that target only one pathway, most often failed in cancer treatment (6, 7). For example, trastuzumab is the first protein kinase inhibitor, which acts on the HER2/neu (erbB2) receptor and is approved for the treatment of cancer (160). However, as for all other kinase inhibitors, it works well only in a small percentage of patients with breast cancer. To improve the efficacy of trastuzumab in breast cancer patients, it will be critical to develop strategies in which it is combined with chemotherapeutic agents or other novel modalities (6). Thus, compounds that can suppress multiple cellular signaling pathways would have great potential in cancer chemoprevention and treatment. Natural compounds may be good sources for this since some of them appear to address multiple targets (6, 7).

Flavonoids, as integral constituents of the diet, are one of the biggest families of natural products and have been proposed to exert beneficial effects in a multitude of disease states, including cancer, cardiovascular disease, and neurodegenerative disorders (79, 161). Classically, many of the biological actions of flavonoids have been attributed to their antioxidant properties, either through their reducing capacities or through their possible influences on intracellular redox status (Fig. 1, left panel). However, recent studies have speculated that their classical hydrogen-donating antioxidant activity (166–168) is unlikely to be the sole explanation for cellular effects (178, 194, 195). For example, the concentrations of flavonoids and their metabolite forms accumulated in the plasma or in organs such as the brain (1), are lower (high nanomolar-low micromolar) than those recorded for small molecule antioxidant nutrients such as ascorbic acid and α-tocopherol (72). Moreover, flavonoids are unlikely to express beneficial action *in vivo* through outcompeting antioxidants such as ascorbate, which are present at higher concentrations (high micromolar) (209).

Recent studies have suggested that the cellular effects of flavonoids may be mediated by their interactions with specific proteins central to intracellular signaling cascades (177, 209, 219). In particular, investigations have indicated that

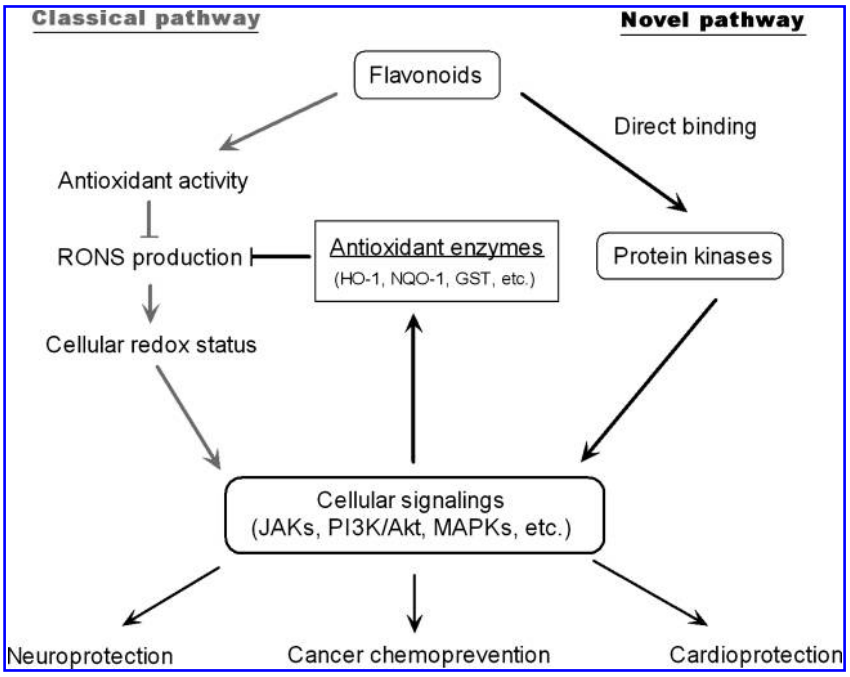

FIG. 1. The classical view of antioxidant effects of flavonoids on cell signaling and responses (*left*). Flavonoids act as antioxidants to modulate the RONS-mediated cellular signaling. The effects of flavonoids on cell signaling and responses by targeting protein kinases (*right*). Flavonoids can bind directly with some protein kinases linking to cellular signaling cascades, and then alter their catalytic activity to regulate multiple cellular signaling pathways. Additionally, flavonoids can also exert their antioxidant properties through binding protein kinases to regulate the expression of antioxidant enzymes, including HO-1, NQO-1, and GSH.

flavonoids may interact selectively with different components of a number of protein kinase signaling cascades such as phosphoinositide 3-kinase (PI3K) (9, 132), Akt/protein kinase B (Akt/PKB) (193), protein kinase C (PKC) (9, 69), and mitogen-activated protein kinase (MAPKs) (110). Some flavonoids can bind these protein kinases directly and alter their phosphorylation state to regulate multiple cell signaling pathways (Fig.1, right panel). Thus, a possible mechanism for the antioxidant functions of flavonoids is that flavonoids may bind directly to some kinases (*e.g.*, MAP kinases), which are central to regulate defense system enzymes, such as heme oxygenase-1 (HO-1) (91), NADPH:quinone oxidoreductase (NQO1) (64), glutathione S-transferase (GST) (172), UDP glucuronosyl transferase (32), thioredoxin (104), heme oxygenase-1 (91), ferritin (203), γ-glutamylcysteine synthetase heavy (or light) subunit (139, 141), metallothionein (164), inducible nitric oxide synthase (116), cysteine/glutamate exchange transporter (175), and Fra-1 (230) (Fig.1, middle). Inhibitory or stimulatory actions at cellular signaling pathways are likely to profoundly affect various cellular functions such as cell defense, neuroprotective, cardioprotective, and cancer chemopreventive actions (Fig. 1).

In this review, we first describe the properties of the binding sites for protein kinase inhibitor and recent methodologies for determining the direct binding and molecular modeling of protein kinase-flavonoids, and then summarize the recent reports of the flavonoids that have been demonstrated to bind with protein kinases directly and/or to dock into the binding site of protein kinases. Flavonoids meeting these criteria include myricetin, quercetin, delphinidin, equol, resveratrol (*trans*-3,5,4′-trihydroxystilbene) analogue (RSVL2), caffeic acid, (-)-epigallocatechin-3-*O*-gallate (EGCG), and procyanidin B2 (Fig. 2) because they have been found to directly bind with protein kinases such as Akt/PKB, Fyn, Janus kinase 1 (JAK1), mitogen-activated protein kinase kinase 1(MEK1), PI3K, mitogen-activated protein (MAP) kinase kinase 4 (MKK4), Raf1, and ζ chain-associated 70-kDa protein (ZAP-70) kinase. These protein kinases are specific proteins essential to intracellular signaling cascades and carcinogenesis. Finally, we discuss the binding sites, selectivity, and the impact on cellular signaling and cancer chemoprevention of flavonoid–protein kinase interactions.

II. Methodologies for Direct Binding Detection and Molecular Modeling of Flavonoid–Protein Kinase

A. Cyanogen bromide–activated Sepharose 4B beads couple with flavonoids

Cyanogen bromide (CNBr)–activated Sepharose 4B is the material of choice for the immobilization of macromolecules in the preparation of biospecific adsorbents for affinity chromatography. Its use in the study of proteins, nucleic acids, and polysaccharides is already well documented (39, 48). Recently, several studies revealed that CNBr-activated Sepharose 4B is able to couple with flavonoids (57, 121). The primary procedures (Fig. 3) contain a) the coupling of flavonoid with CNBr-activated Sepharose 4B beads in a coupling buffer; b) the binding of the coupled flavonoid-Sepharose 4B beads with cell lysates or recombinant protein kinase in a reaction buffer; c) washing the binding complex with buffer to move nonspecific binding; and d) the detection of proteins bound to flavonoid-Sepharose 4B beads by immunoblotting assay with specific antibody. This procedure enables the identification of kinases directly interacting with flavonoids without any further information on binding affinity or the functional category of the binding sites. Therefore, it provides a very convenient way to screen and identify the direct binding of flavonoids to proteins.

On the other hand, it is noteworthy about the change in surface properties of CNBr-activated Sepharose 4B after coupling with flavonoids, for example, the hydrophobic or hydrophilic and the electrostatic properties of the bead surface, which are key factors that affect the absorption of biological molecules such as proteins. Most flavonoids have

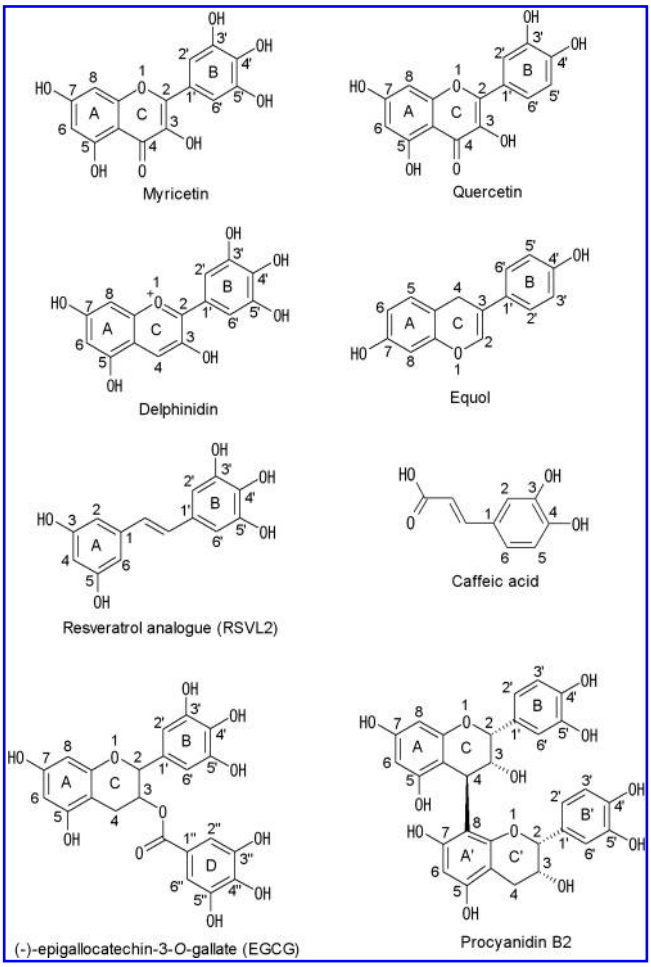

FIG. 2. Chemical structures of flavonoids reviewed in this article.

been found to couple with CNBr-activated Sepharose 4B, suggesting that CNBr-activated Sepharose 4B is a common coupling beads for flavonoids. However, whether any kind of these flavonoids coupled with Sepharose 4B can interact with the interested proteins is not clear. Thus, Sepharose 4B alone used as negative control is not appropriate any longer, although it was used in some experiments. A negative protein is required for control. As shown in Figure 3A, myricetin coupled by CNBr-activated Sepharose 4B could bind to JAK1, Akt and MEK1, but did not bind to EGFR.

B. Affinity analysis of flavonoid–protein kinase interaction

There are two general technologies that have been reported for analyzing the interactions of protein and flavonoid. One is surface plasmon resonance (SPR) technology. Another is quartz-crystal microbalance (QCM) technology. Both technologies can detect the interactions between protein and flavonoid as a label-free manner.

1. SPR. SPR is an optical technology that uses the evanescent wave phenomenon to measure changes in refractive index very close to a sensor surface. The binding of an analyte in solution with its ligand immobilized on the sensor surface results in a change in the refractive index. The interaction is monitored in real time and the amount of bound ligand and rates of association and dissociation can be measured with high precision. Thus, SPR is a powerful and versatile spectroscopic method for biomolecular interaction analysis (BIA) and has been well reviewed in previous years (101, 135, 159). In the case of Biacore X (GE Healthcare UK Ltd, Chalfont, United Kingdom), protein was immobilized on a CM5 sensor chip (research grade) using the amino coupling kit. Aliquots of 40 μl of flavonoids in Hepes-EDTA buffer were injected in the flow cell. Affinity between flavonoids and protein was analyzed by BIA evaluation software 4.0 (187). It has been reported that quercetin and its metabolites can bind to myeloperoxidase (MPO) as inhibitors (187), using this SPR method. Moreover, the cells also can be immobilized on such sensor chips to detect the interaction of flavonoid–cell surface (or membrane receptor). For example, the interaction between EGCG and human basophilic KU812 cells was investigated, using SPR biosensor SPR670. The cells were immobilized on the sensor chip. EGCG was diluted at 25 μM in running buffer in 60-μl injection volumes and added at a flow rate of 30-μl/min. Binding was measured at 25°C for 2 min. The value of resonance unit is corresponded with the binding strength (68). It has reported that EGCG can bind to 67-lamin receptor with nM dissociation constant (Kd) value by SPR analysis (199).

2. QCM. QCM is a nanogram-sensitive technology that utilizes acoustic waves generated by oscillating a piezoelectric, single crystal quartz plate to measure mass. The basis of QCM operation relates to quartz's inherent property of piezoelectricity. Piezoelectricity stems from the Greek word piezin which means to press and the electricity that is generated by the pressure (22). QCM technology in nonbiological applications has been well known for many decades. The core component of the device is a thin quartz disk that is sandwiched between two evaporated metal electrodes, and is commonly referred to as a thickness shear mode (TSM) resonator (43). Since oscillator circuits capable of exciting shear vibrations of quartz resonators under liquid loading have been developed, the QCM is accepted as a new powerful technology to follow adsorption processes at solid–liquid interfaces in chemical and biological research. In recent years, there has been a rapid growth in the number of scientific reports. QCM technology has played a key role in elucidating various aspects of biological materials and their interactions, including small molecular weight ligands, carbohydrates, proteins, nucleic acids, viruses, bacteria, cells, and lipidic and polymeric interfaces (43, 54, 214).

In our laboratory, we used a QCM AFFINIX Q instrument (Initium, Tokyo, Japan) to investigate the affinity binding activity between flavonoids and protein kinases since this instrument is a very sensitive mass-measuring device. The resonance frequency is decreased linearly with the increasing mass on the QCM electrode at a nano gram level. Thus, it is used to investigate molecule interactions in aqueous solutions. The change in resonance frequency is recorded in real time as delta F (Hz). The rate of delta F (Hz) is dependent on both the amount of immobilized kinase and the concentration of flavonoids. Thus, it is essential to optimize the amount of immobilized kinase before screening the interaction of flavonoids–kinase, and to monitor a dose-dependent delta F by different concentrations of flavonoids. Kd constant is ob-

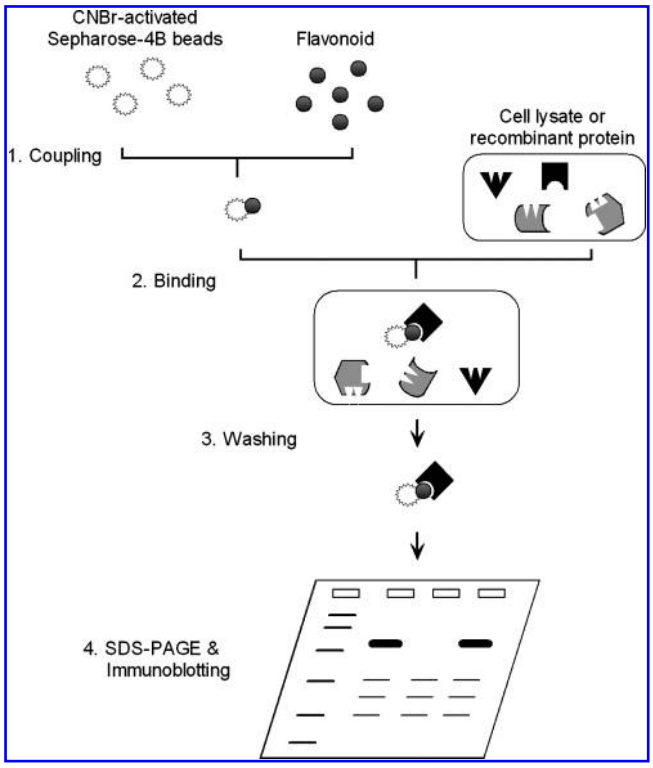

FIG. 3. Pull-down assay for detecting flavonoid–protein kinase binding. The procedures contain (1) coupling flavonoid with CNBr-activated Sepharose 4B beads in a coupling buffer [0.5 *M* NaCl and 35% DMSO (pH 8.3)] overnight at 4°C; (2) binding the coupled flavonoid-Sepharose 4B beads with cell lysates or recombinant protein kinase in a reaction buffer [50 m*M* Tris (pH 7.5), 5 m*M* EDTA, 150 m*M* NaCl, 1 m*M* DTT, 0.01% Nonidet P-40, 2 μg/ml BSA, 0.02 m*M* PMSF, and 1 μg protease inhibitor cocktail); (3) washing the binding complex with 50 m*M* Tris (pH 7.5) buffer containing 5 m*M* EDTA, 200 m*M* NaCl, 1 m*M* DTT, 0.02% Nonidet P-40, and 0.02 mm PMSF; (4) the proteins bound to the beads are applied to SDS-PAGE and then detected by immunoblotting assay with specific antibody.

tained by plotting the rate of steady-state delta F vs flavonoid concentration, which can be calculated by software. A sample procedure is shown in Figure 4.

Although many fundamental differences exist between QCM and SPR technologies, there are nevertheless several similarities between the physical principles of QCM and SPR sensors (165). The QCM setup is relatively simple and less expensive than SPR, but the frequency measurement is more environmentally sensitive. SPR has probably better reproducibility and reliability; however, relatively complex and expensive instruments are needed (165).

3. [^{3}H]-labeled flavonoids. Recent studies with a [^{3}H]-labeled EGCG showed that EGCG could bind with vimentin (57), GRP-78 (58), Fyn (76), and ZAP-70 (188) proteins. The target protein kinase was expressed by GST-fusion protein and then incubated for immobilization with glutathione-Sepharose 4B beads for 1 h at room temperature. The affinity binding assay was carried out overnight at 4°C in a 500 μl reaction mixture containing reaction buffer [50 m*M* Tris, pH 7.5, 5 m*M* EDTA, 150 m*M* NaCl, 1 m*M* DTT, 0.01% Nonidet P-40, 2 μg/ml BSA, 0.02 m*M* PMSF, 1× protease inhibitor mixture] with 1 μg of GST-fusion protein-Sepharose 4B or GST-Sepharose 4B and 0.5 μCi of [^{3}H] EGCG. For analyzing concentration-dependent uptake, 39 p*M* to 50 μ*M* concentrations of cold EGCG were applied. The Kd was determined through nonlinear regression analysis using the Prizm 4.0 software program (Graphpad Inc., San Diego, CA).

Kd represents the affinity between a flavonoid and a protein kinase. The smaller Kd, the higher the affinity between flavonoid and protein kinase is. Since flavonoids are commonly pumped out of cells by efflux transporters, the intracellular concentration of flavonoid is very low, ranging from n*M* to low μ*M* (79) even when administered at therapeutic doses. Moreover, ATP can saturate the kinase catalytic site to compete with flavonoids for binding. Thus, Kd assessing is a very important primary step to screen the flavonoid–protein kinase interaction in practice. A small Kd will be a highly predictive of *in vivo* bioactivity of flavonoid.

C. Molecular modeling of flavonoid–protein kinase docking

The X-ray crystallographic structures of protein kinase bound to its inhibitors is important to identify their interaction and to design more specific inhibitors. On the other hand, the crystallization of protein kinase and its inhibitors depend on the properties of both protein kinase and inhibitor. It is hard to obtain the crystal in some cases. Thus, three-dimensional (3D) pharmacophore modeling technology has been developed to describe the interaction of a small molecule ligand with a macromolecular target such as protein. Since chemical features in a pharmacophore model are well known and highly transparent for medicinal chemists, these models are intuitively understandable and have been increasingly successful in computational drug discovery in the past few years (111, 222). Latest developments in 3D pharmacophore modeling packages involve Catalyst (106, 156a), Molecular Operating Environment (MOE) (140), Phase (55, 157), and LigandScout (220, 221). The quantitative structure–activity relationship (QSAR) has been developed to investigate the inhibitory effects of protein kinase by flavonoids (75). Several studies have reported the inhibitory activity of flavonoids against protein kinases, using different descriptors and methods of modeling (62, 50, 148).

Here, we show the key technologies and steps of MOE modeling packages (MOE, Version 2008.10, Chemical Computing Group Inc., Montreal, Canada). MOE can perform molecular modeling and simulations, cheminformatics and high throughput discovery, pharmacophore modeling, protein modeling and bioinformatics, and structure-based drug design. In our laboratory, we search for the flavonoid–protein kinase binding site using MOE modeling package. Figure 5 shows the primary procedures for molecular modeling, using Akt1 and myricetin as examples. We first download the crystal structure of the interested protein kinase (*e.g.*, Akt1) from Protein Data Bank (http://www.rcsb.org/pdb/) (step 1), and then delete crystallization water and other molecules involved in protein kinase structure (step 2) due to crystallization water exist in the X-ray crystallization of protein kinase. At step 3, protein kinase structure is calculated for hydrogenation, structure optimization, and forcefield setup (MMFF94x), which is suitable to investigate flavonoid–protein kinase docking. The candidate sites for ligand-binding

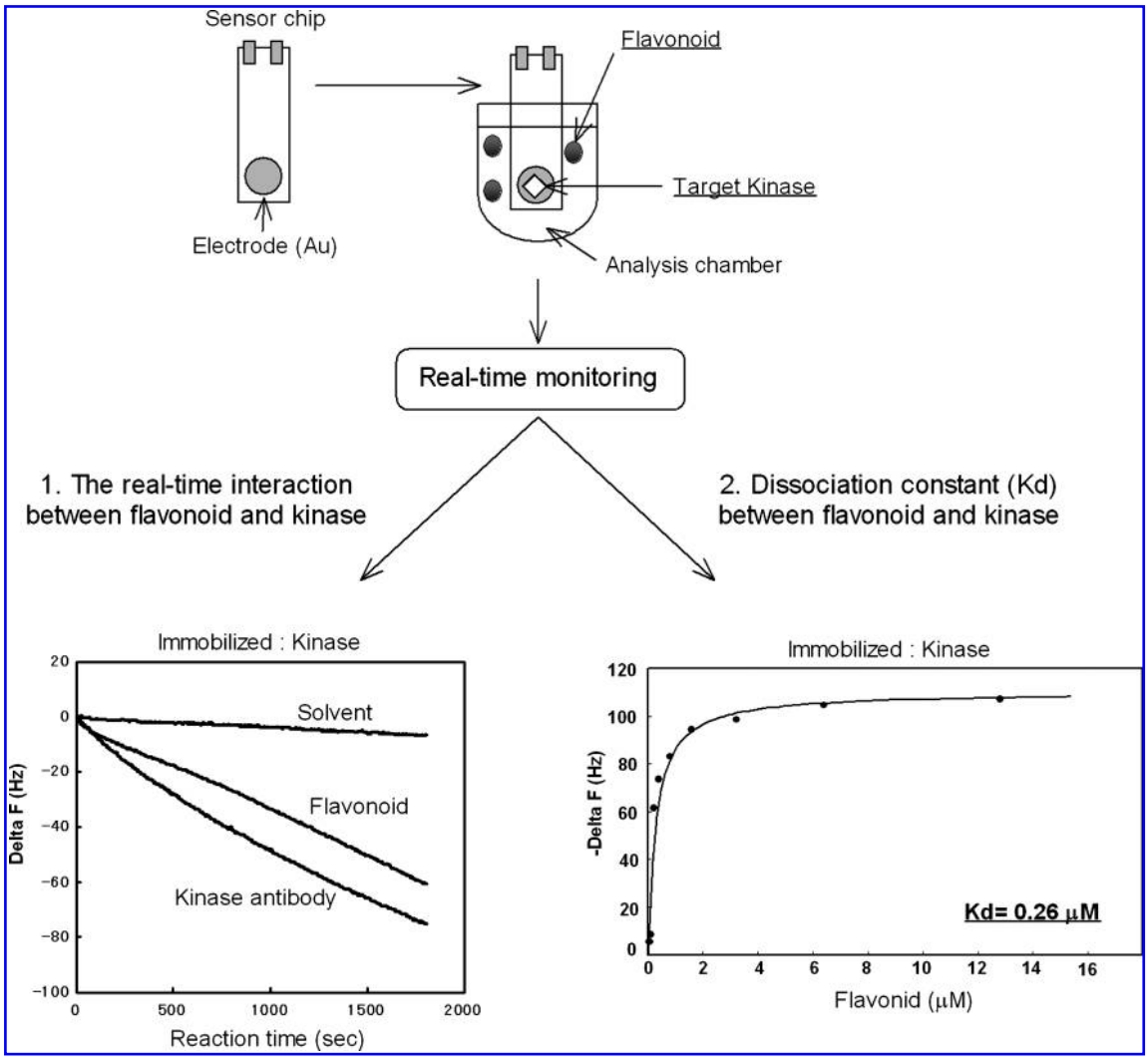

FIG. 4. Affinity assay of flavonoid–protein kinase interaction, using QCM. The recombinant protein kinase (100 ng) is immobilized into the QCM electrode plate for 1 h at room temperature. After washing 5 times with a binding buffer [50 m*M* Tris-HCl, 150 m*M* NaCl, 1 m*M* EDTA, 2 μg/ml BSA, pH 8.0], electrode plate was soaked in the binding buffer at 25°C. Solvent flavonoid was applied to the equilibrated solution. Flavonoid is then injected stepwise into the analysis chamber. (1). The change in resonance frequency is real-timely recorded as delta F (Hz) (*left panel*), and (2) Kd constant is obtained by plotting the rate of steady-state delta F vs. flavonoid concentration, which can be calculated by a software programm (AFFINIX, Japan).

sites will automatically found on target protein kinase surface (step 4).The docking between candidate sites and flavonoid is then simulated with ASEDock 2005 software (step 5). It has four stages that consist of conformation search of the ligand, assembly of ASEModel, posing, and optimization calculation of energy. Finally, the molecular modeling for flavonoid–protein kinase is established according to the docking data (step 6). The modeling data calculated by MOE software support the flavonoid–protein kinase binding complex obtained by pull-down and affinity assay. Moreover, the molecular modeling of flavonoid–protein kinase will suggest their detail binding sites and three-dimensional structure.

III. Direct Binding and Molecular Modeling of Flavonoids to Protein Kinases

Accumulated data have indicated that flavonoids might exert their bioactivity by modulating intracellular signaling cascades. In particular, some flavonoids have been demonstrated to interact directly with protein kinases, which play a key role in intracellular signaling cascades and carcinogenesis. In this section, we review those flavonoids that have been demonstrated to bind directly with protein kinases and/or to dock into the specific binding site of protein kinases. The detailed information is reported according to flavonoids, including myricetin, delphinidin, EGCG, quercetin, caffeic acid, equol, resveratrol analogue (RSVL2), and procyanidin B2. The direct binding and molecular modeling of these flavonoid–protein kinases are summarized in Table 1.

A. Myricetin–JAK1, Akt, MEK1, Fyn, MKK4, and PI3K

Myricetin is an abundant natural flavonoid found in fruits, vegetables, and common drinks such as red wine, garlic, guava, and onion juice. Myricetin has been reported to have many biological activities (151). Recently, the fol-

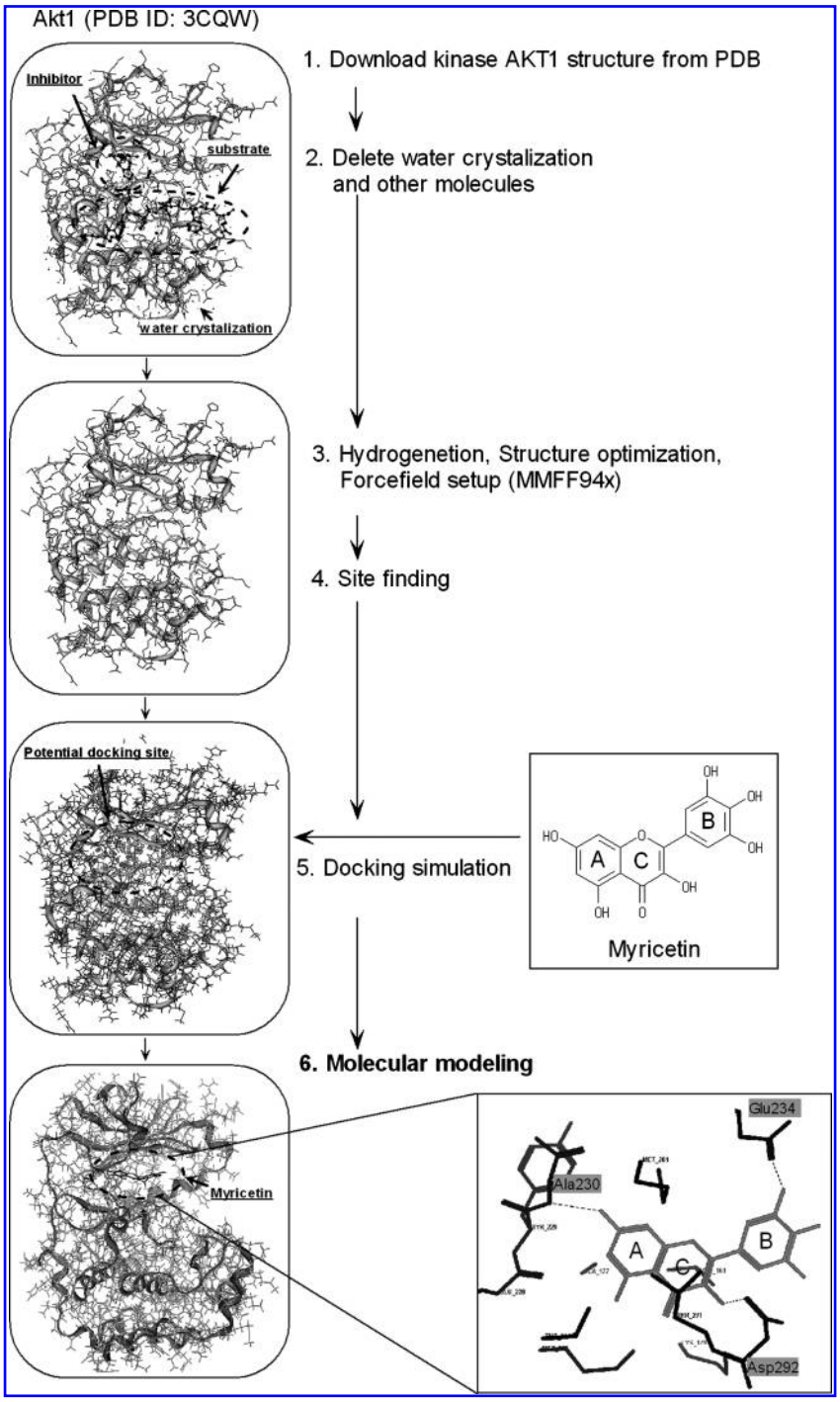

FIG. 5. Molecular modeling of flavonoid–protein kinase binding, using MOE software. Protein kinase Akt and flavonoid myricetin are used here as examples. The procedures contain (1) downloading the three-dimensional structure of the interested protein kinase (Akt) from PDB; (2) deleting the water crystallization and/or other molecules involved in protein kinase structure; (3) performing a hydrogenation, structure optimization, and forcefield setup (MMFF94x); (4) finding the candidate sites for ligand-binding sites on the target protein kinase surface; (5) docking flavonoid (myricetin) into the candidate sites on the target protein kinase with ASEDock 2005 software, which includes conformation search of the ligand, assembly of ASEModel, posing, and optimization calculation of energy; (6) molecular modeling based on the docking data of flavonoid (myricetin) into protein kinase (Akt1). The three-dimensional structure of Akt1 is obtained from PDB (ID: 3CQW).

lowing protein kinases have been reported to bind with myricetin.

1. JAK1. The JAK family is one of nonreceptor tyrosine protein kinases and plays a critical role in cellular survival, proliferation, differentiation, and apoptosis. It is known that JAK kinase is required for the tyrosine phosphorylation of signal transducers and activators of transcription 3 (STAT3), which is activated by cytokines and growth factors (162). Myricetin is found to bind with JAK1. The Sepharose 4B-myricetin-coupled beads bind with JAK1 either from purified recombinant fusion GST-JAK1 (*in vitro*) or from mouse JB6 cell lysates (*ex vivo*) (Fig. 6A) (112). Furthermore, a higher affinity of myricetin to JAK1 was observed in a QCM electrode plate immobilized by JAK1 (kinase domain) after adding 10 μM and 20 μM of myricetin (Fig. 6B). Myricetin showed rapid formation of the JAK1–myricetin complex. On the contrary, piceatannol, which is a specific inhibitor for JAK1, slowly reacts with JAK1, similar to the slow formation of JAK1–antibody complex. The affinities of 20 μM myricetin and 25 μM piceatannol to JAK1 became similar at 30 min. Additional cell signaling data further supported that the direct binding of JAK1 by myricetin downregulated downstream molecular events including epidermal growth factor

Table 1. Summary of Direct Binding and Docking information of Flavonoid–Protein Kinase

			Molecular modeling			
Flavonoid	*Kinase*	*Binding analysis*	*Binding site*	*Hydrogen bond OH position-(amino acid residue)*	*Hydrophobic interaction*	*Reference*
Myricetin	JAK1	Pull down (*In vitro*, *ex vivo*) Affinity assay	n.d.	n.d.	n.d.	[89]
Myricetin	Akt	Pull down (*In vitro*, *ex vivo*) Affinity assay ($Kd = 0.26\,\mu M$)	ATP-binding site	3′-(Glu234), 3-(Asp292), 7-(Ala230)	n.d.	[90]
Myricetin	MEK1	Pull down (*In vitro*, *ex vivo*)	n.d.	n.d.	n.d.	[97]
Myricetin	Fyn	Pull down (*In vitro*, *ex vivo*)	ATP-binding site	3′-(Glu342, Thr341), 4′-(Met344), 5-(Asn278), 7-(Glu3 13)	Leu276, Val284, Ala296, Val326, Leu396, Ala406	[73]
Myricetin	MKK4	Pull down (*In vitro*, *ex vivo*)	ATP-binding site	3′-(Glu179), 4-(Ser233), 5-(Lys187), 4′-(carbonyl and amide groups of Met181)	Ile108, Val1 16, Ala120, Cys156, Met178, Met181, Leu236	[81]
Myricetin	PI3Kγ	X-ray crystallographic structure	ATP-binding site	3′, 4′-(Val882), 4, 5-(Lys833), 5, 7-(Asp964), 7-(Tyr867)	n.d.	[172]
Delphinidin	Raf-1	Pull down (*In vitro*, *ex vivo*)	Activation loop	4′-(Thr598), 5′-(Gly595), Other 3 sites	2 sites	[74]
Delphinidin	MEK1	Pull down (*In vitro*, *ex vivo*)	Activation loop	3′-(Ser2 12), 7-(Val127)	5 sites	[74]
Delpinidin	Fyn	Pull down (*In vitro*, *ex vivo*)	*Allosteric site*	4′-(Tyr343), 3-(Met249), 5-(Gln1 61)	Met344, Ile402	[66]
EGCG	ZAP-70	Pull down (*In vitro*, *ex vivo*) Affinity assay ($Kd = 0.6207\,\mu M$)	ATP-binding site	3′-(Glu386), 4′-(Lys369), 5′-(Asp479), 7-(Glu415, Ala417))	n.d.	[155]
EGCG	Fyn	Pull down (*In vitro*, *ex vivo*) Affinity assay ($Kd = 0.367\,\mu M$)	n.d.	n.d.	n.d.	[59]
Quercetin	MEK1	Pull down (*In vitro*, *ex vivo*)	Activation loop	3′-(Ser2 12), 7-(Val127)	Ile99, Leu1 18, Ile141, Phe209, Val211, Leu215	[96]
Quercetin	PI3Kγ	X-ray crystallographic structure	ATP-binding site	4′-(Lys833), 3-(Glu880), 4-(Ile881), 5- (Val882)	n.d.	[172]
Caffeic acid	Fyn	Pull down (*In vitro*, *ex vivo*)	ATP-binding site	3-(Tyr343), 4-(Asn345)	(Salt bridge between COOH and Arg160)	[77]
Equol	MEK1	Pull down (*In vitro*, *ex vivo*)	Activation loop	7-(Val127)	Leu118, Ile141, Met143, Phe209, Val211, Leu215, Met219	[76]
Resveratrol analogue (RSVL2)	MEK1	Pull down (*In vitro*, *ex vivo*)	Activation loop	3′-(Ser2 12), 3-(Val127)	Ile99, Ile1 14, Leu1 15, Val127, Met143, Phe209, Val211, Leu215	[98]
Procyanidin B2	MEK1	Pull down (*In vitro*, *ex vivo*)	n.d.	n.d.	n.d.	[75]
Procyanidin B2	MEK4	Pull down (*In vitro*, *ex vivo*)	n.d.	n.d.	n.d.	[32]

n.d., not determined.

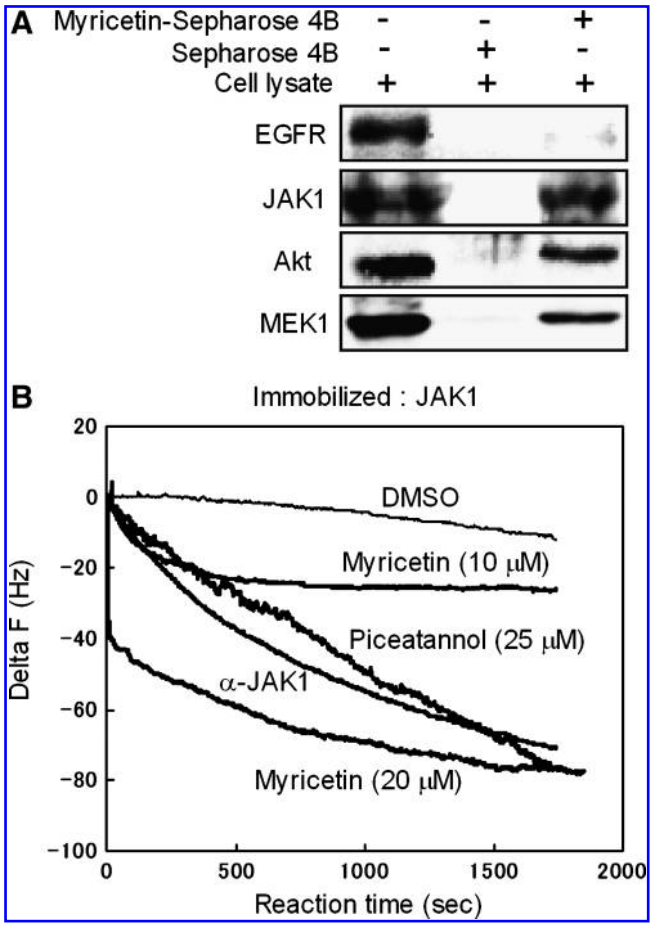

FIG. 6. Myricetin binds directly to JAK1, Akt, and MEK1. (**A**) *Ex vivo* pull-down assay. Three mg of myricetin was coupled to CNBr-activated Sepharose 4B beads (25 mg) in a coupling buffer [0.5 *M* NaCl and 35% DMSO (pH 8.3)] overnight at 4°C. The mixture was washed in 5 volumes of coupling buffer and then centrifuged at 1000 rpm for 3 min at 4°C. The precipitate was resuspended in 5 volumes of 0.1 *M* Tris-HCl buffer (pH 8.0) with 2 h rotation at room temperature. After washing three times with 0.1 *M* acetate buffer (pH 4.0) containing 0.5 *M* NaCl, the mixture was further washed with 0.1 *M* Tris-HCl (pH 8.0) buffer containing 0.5 *M* NaCl. Mouse JB6 cell lysate (500 μg) was incubated at 4°C overnight with Sepharose 4B beads or Sepharose 4B-flavonoid-coupled beads (100 μl, 50% slurry) in a reaction buffer [50 m*M* Tris (pH 7.5), 5 m*M* EDTA, 150 m*M* NaCl, 1 m*M* DTT, 0.01% Nonidet P-40, 2 μg/ml BSA, 0.02 m*M* PMSF, and 1 μg protease inhibitor cocktail] (Fig. 3, Binding). The beads were then washed 5 times with 50 m*M* Tris (pH 7.5) buffer containing 5 m*M* EDTA, 200 m*M* NaCl, 1 m*M* DTT, 0.02% Nonidet P-40, and 0.02 m*M* PMSF. Whole cell lysate (input control, *lane 1*), lysates with Sepharose 4B beads alone (negative control, *lane 2*) or with Sepharose 4B-myricetin-coupled beads were applied to SDS-PAGE, and detected with antibody against EGFR, JAK1, Akt or MEK1, respectively, after transferring to membrane. (**B**) Affinity assay of myricetin (10–20 μM) to JAK1 by QCM. GST-tagged JAK1 was immobilized on the electrode. DMSO was used as a solvent control. JAK1 antibody was used as a positive control. Piceatanol (25 μM) is a JAK1-specific inhibitor and used to compare with myricetin. The interaction of flavonoids with the kinase is detected as Delta F. The affinity is expressed as Kd, which is calculated, using a given equation.

(EGF)-induced STAT3 phosphorylation, DNA binding, and cell transformation (112).

2. Akt. Akt, a Ser/Thr kinase, is a critical regulator in many cellular processes, including cell growth, proliferation and apoptosis (78). Up to date, more than 50 proteins have been identified as phosphorylation substrates of Akt. Of these, transcription factor, activator protein-1 (AP-1), nuclear factor-kappa B (NF-κB), and cyclic AMP (cAMP)-response element binding protein (CREB), have been identified as the primary targets of Akt signaling. Myricetin has been reported to directly target Akt to inhibit cell transformation (113). *Ex vivo* and *in vitro* binding data showed that myricetin directly bound to the ATP-binding site of Akt. Affinity assays revealed a higher binding affinity of myricetin to Akt (Kd = 0.26 μM). Molecular modeling data revealed that myricetin docked to the pocket of the ATP-binding site, which is located in the middle of the hinge connecting the N and C-lobe of Akt (Fig. 7). Three hydrogen bonds were formed between the 3′, 3, and 7 positions of myricetin and Glu234, Asp292, or Ala 230 residues of Akt, which configured the binding pocket. When myricetin is superposed on Akt, the hydrophobic surface was formed at the pocket of ATP-binding site (Fig. 7). Additional cell signaling data further supported that the direct binding and kinase activity inhibition of Akt by myricetin downregulated downstream molecular events including 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced AP-1 activation, cyclin D1 expression, cell cycle progression, and cell transformation (113).

3. MEK1. MEK1 is a member of a dual-specificity family of protein kinases and phosphorylate extracellular signal-regulated kinase (ERK) 1/2 substrates. MEK1 is essential for integrating signals into the MAPK pathway, and is an important regulator of various cellular processes, including proliferation, migration, and survival (37, 169). *Ex vivo* binding data showed that myricetin directly bound with MEK1 (121). ATP addition did not compete with myricetin for binding with MEK1 *in vitro*, suggesting that the binding of myricetin to MEK1 was distinct from the ATP binding site. Pull-down assay of Sepharose 4B-myricetin-coupled beads with three MEK1 deletion mutants further identified that myricetin directly bound to MEK1 without competing with ATP. Additional cell signaling data revealed that the direct binding of MEK1 by myricetin downregulated downstream signaling to the ERK/p90 ribosomal S6 kinase (p90RSK)-AP-1 pathway and inhibited cell transformation (121).

4. Fyn. Fyn is a ubiquitously expressed member of the Src family of nonreceptor tyrosine kinases that are involved in transmitting signals from various cell surface receptors to cytoplasmic signal transduction cascades. Fyn comprises four domains: an N-terminal membrane-anchoring domain, a Src homology 3 (SH3) domain, a SH2 domain, and a catalytic kinase domain. The kinase domain of the enzyme consists of a N-lobe and a C-lobe. The N- and C-lobes are linked through a loop, which is referred to as the 'hinge region,' and the ATP-binding site is flanked by these two lobes. The backbone of this loop interacts with the adenine moiety of ATP by hydrogen bonding. Fyn plays a critical role in T cell receptor signaling, brain function, and cell adhesion-mediated signaling, and cell transformation (173). Myricetin was found to

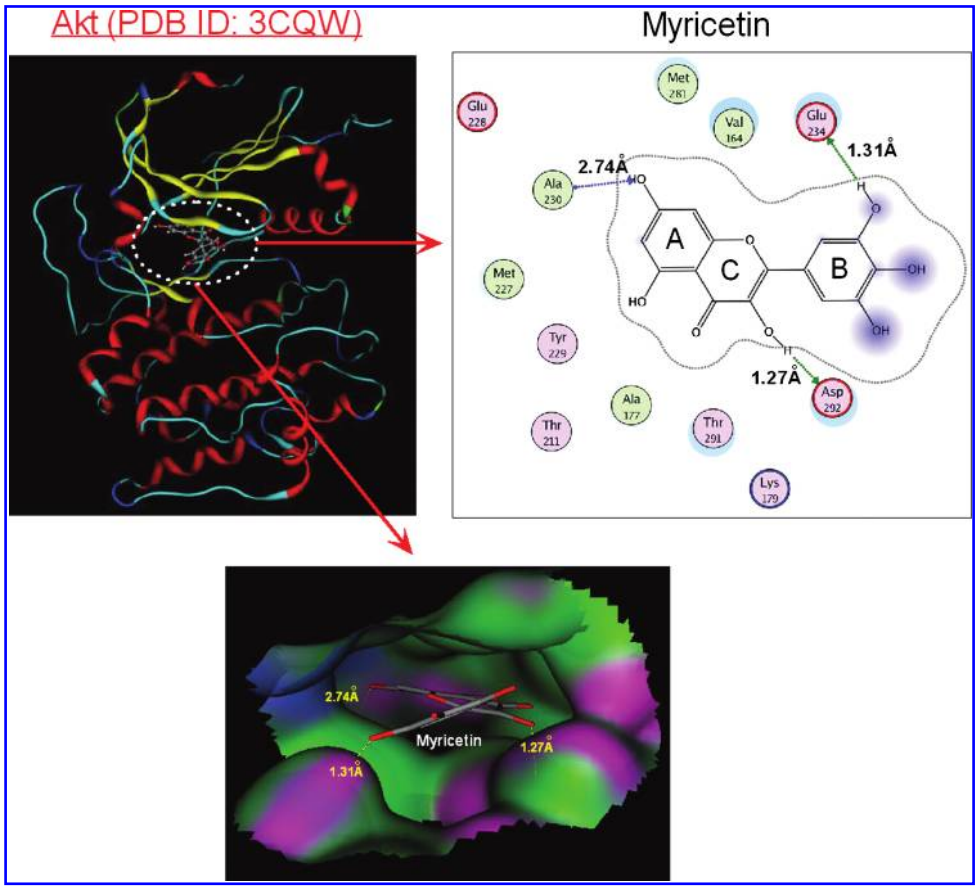

FIG. 7. Molecular modeling of myricetin binding to Akt. (*Left*) Myricetin docks to the pocket of Akt ATP-binding site, which is located in the middle of the hinge connecting the N- and C-lobes. (*Right*) Three hydrogen bonds are formed between the 3′, 3, and 7 positions of myricetin and Glu234, Asp292, or Ala230 residues of Akt, which configured the binding pocket. *Red*: α-helix, *yellow*: β-sheet, *blue*: 3-turn, *yellow–green*: 4- or 5-turn, and *aqua*: loop in left panel. Hydrogen bonds are indicated by *blue* (to backbone) and *green* (to sidechain) lines. (*Bottom*) Myricetin is superposed on the ATP-binding site of Akt. The hydrophobic surface is formed at the pocket. *Pink*: hydrogen bonds; *Green:* hydrophobic; *Blu:* Mild polar surfaces. Hydrogen bonds are indicated by *yellow line*. (Partially modified from Ref. 112.) (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

directly bind to the ATP-binding site of Fyn (95). Modeling data showed that myricetin is easily docked to the Fyn ATP-binding site, which is located between the N and C-lobes of the kinase domain (Fig. 8C). Myricetin can form hydrogen bonds with the backbone of the hinge region of Fyn. The hydroxyl group at the 4′ position works as a hydrogen bonding acceptor in the interaction with the backbone amide group of Met344, and the hydroxyl group at the 3′ position is a hydrogen bonding donor in the interaction with the backbone carbonyl group of Glu342. At the same time, it can also work as a hydrogen bonding acceptor to interact with the side chain of Thr341. The hydroxyl group at the 7 position would make a hydrogen bond with the side chain of Glu313 and the hydroxyl group at the 5 position could also form a hydrogen bond with the backbone carbonyl group of Asn278 (Fig. 8C). In addition, the inhibitor would be sandwiched by hydrophobic residues, including Leu276, Val284, and Ala296 from the N-lobe, and Val326, Leu396, and Ala406 from the C-lobe, and thereby lead to a high inhibitory activity for Fyn (Fig. 8C). Cell signaling data further revealed that the direct binding of Fyn by myricetin attenuated ultra violet B (UVB)-induced phosphorylation of MAPKs, transactivation of AP-1 and NF-κB, and expression of cyclooxygenase-2 (COX-2) (95).

5. MKK4. MKK4 is a component of stress-activated MAP kinase signaling modules, and is expressed ubiquitously. It directly phosphorylates and activates the c-Jun N-terminal kinase (JNK) and p38 families of MAP kinases in response to environmental stress, pro-inflammatory cytokines, and developmental cues (218). *Ex vivo* and *in vitro* binding data revealed that myricetin could bind to the ATP-binding site of MKK4 (103). The docking data revealed that myricetin fits easily onto the ATP-binding site of MKK4, located between the N- and C-lobe of the kinase domain. Myricetin can form hydrogen bonds with the backbone of the hinge region in MKK4, as do other ATP-competitive kinase inhibitors. The hydroxyl group at the 3′ position acts as a hydrogen bond donor in the interaction with the carbonyl of Glu179. The hydroxyl group at the 4′ position functions as a hydrogen bond donor in the interaction with the carbonyl of Met181, and also can function as a hydrogen bond acceptor in the interaction with the amide group of Met181. The carbonyl group at position 4 and the hydroxyl group at position 5 for hydrogen bonds with the side chains of Ser233 and Lys187, respectively. In addition, the inhibitor is sandwiched by the side chains of the hydrophobic residues in the ATP-binding site (*e.g.*, Ala120, Met178, Ile108, Val116, Cys156, Leu236, and Met181). The surface of the putative myricetin-binding site in MKK4 accommodates the compound without any steric collision, leading to the high level of inhibitory activity against MKK4. Cell signaling data supported that the direct binding of MKK4 by myricetin attenuated tumor necrosis factor-alpha (TNF-α)-induced JNK and ERK phosphorylation, AP-1 activation, and vascular endothelial growth factor (VEGF) expression (103).

6. PI3K. PI3K is a lipid kinase that phosphorylates phosphoinositides at the 3-hydroxyl. We enclose it in this review since PI3K is involved in a large number of funda-

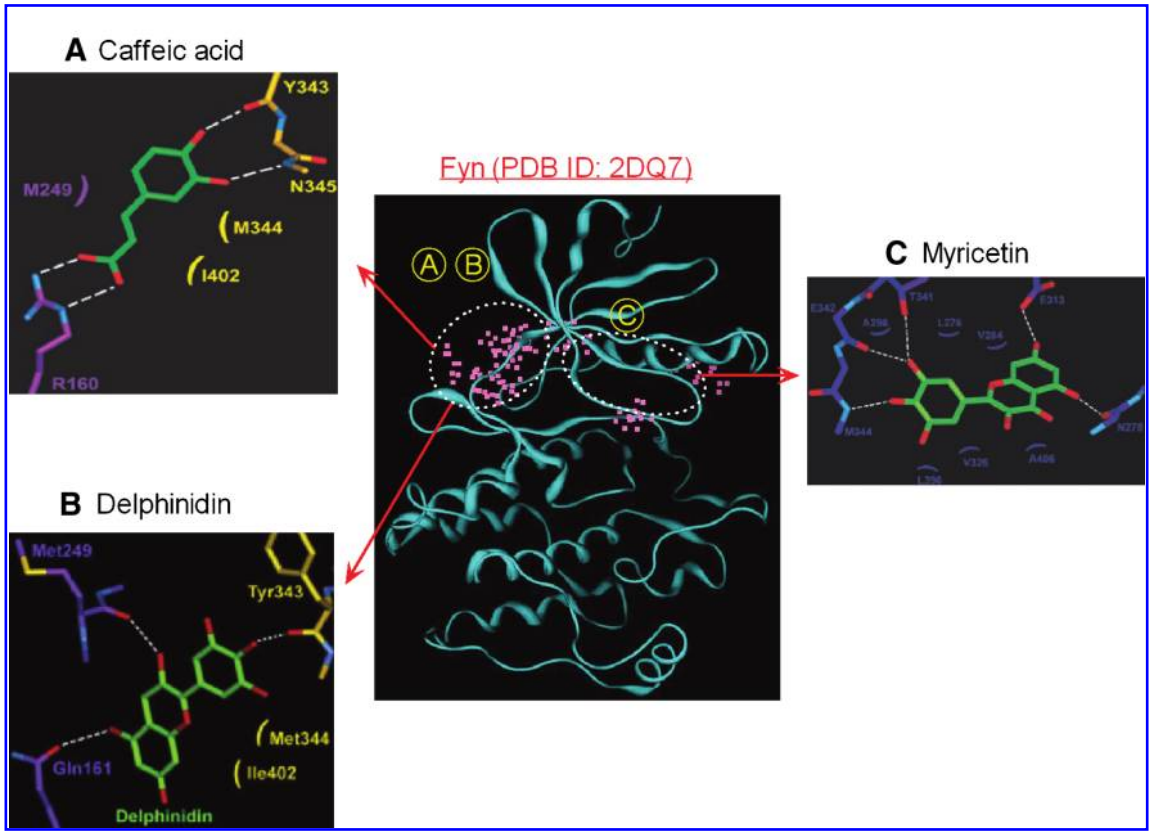

FIG. 8. Molecular modeling of caffeic acid, delphinidin, and myricetin binding to Fyn. (*Middle*) The tube model of Fyn structure from PDB (ID: 2DQ7). *Pink points* show the direct binding sites of these flavonoids with Fyn. *White ellipse* represents the binding region of flavonoids with Fyn. (**A**) Caffeic acid–Fyn docking. *Green line*: caffeic acid; *Purple line*: backbone of Fyn; *Yellow line*: activation loop site of Fyn; *Arc line*: *van der waals* interactions of caffeic acid to Fyn. Hydrogen bonds are indicated by *white line* (99). (**B**) Delphinidin–Fyn docking. *Green line*: delphinidin; *Purple line*: backbone of Fyn; *Yellow line*: activation loop site of Fyn; *Arc line*: *van der waals* interaction of delphinidin to Fyn. Hydrogen bonds are indicated by *white line* (88). (**C**) Myricetin–Fyn docking. *Green line*: myricetin; *Purple line*: backbone of Fyn; *Arc line*: *van der waals* interactions of myricetin to Fyn. Hydrogen bonds are indicated by *white line* (95). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

mental cellular processes, including apoptosis, proliferation, cell motility and adhesion, which in turn are involved in cancer (71). PI3K is a heterodimer that consists of a p85 regulatory subunit and a p110 catalytic subunit. The X-ray crystallographic structure of PI3Kγ bound to myricetin revealed that myricetin fits into the ATP-binding pocket with a Kd of 0.17 μM, which is slightly lower than that of quercetin. The chromone moiety of myricetin interacts with the side chains of Asp964, Tyr967, and Lys833 (211).

B. Delphinidin–Raf1, MEK1, Fyn

Delphinidin is a representative aglycone of dietary anthocyanins, which naturally occur in many fruits and vegetables with intense color such as berries, red grapes, purple sweet potato, and red cabbages (81). Accumulated data showed that anthocyanidins scavenge reactive oxygen species (ROS) (81), suppress cell transformation (83), inflammation (82, 85), tumor cell invasion and angiogenesis (95, 213), and induce apoptosis of cancer cells (84).

1. Raf1 and MEK1. Raf1 is a Ser/Thr protein kinase, and intimately involved in the transmission of cell regulatory signals controlling proliferation and differentiation. The best characterized Raf substrates are MEK1 and MEK2 (153). The Sepharose 4B-delphinidin-coupled beads bound with Raf1 and MEK1 without competing ATP (96). Modeling data showed that delphinidin is docked to the pocket separate from the ATP-binding site by several hydrogen bonds and hydrophobic interactions. Especially, the C-ring moiety of delphinidin forms hydrogen bonds with the backbone carbonyl group of Gly595 and the side chain of Thr598 of the activation loop of Raf1 and thereby can stabilize the inactive conformation of the activation loop of B-Raf. Interestingly, delphinidin could be also docked to the pocket separate from but adjacent to the ATP-binding site of MEK1 (Fig. 9A). The interactions by the C-ring moiety are critical for the holding of the activation loop of the inactive MEK1. The inactive conformation of the activation loop can be stabilized by the hydrophobic interactions of Val211 and Leu215, and the hydrogen bond of the backbone amide group of Ser212 with the C-ring of delphinidin (Fig. 9A). Additional investigation on cell signaling further revealed that the direct binding of Raf1 and MEK1 by delphinidin downregulated the phosphorylation of TPA-induced MEK1, ERK, p90RSK, and mitogen- and stress-activated protein kinase (MSK). Consequently,

delphinidin inhibited TPA-induced AP-1 and NF-κB activation, COX-2 expression, and cell transformation.

2. Fyn. *Ex vivo* and *in vitro* binding data revealed that delphinidin also directly bound to Fyn kinase noncompetitively with ATP (88). Docking data revealed that delphinidin is docked to the putative allosteric site of Fyn kinase (Fig. 8B). In the hypothetical structure of the Fyn kinase–delphinidin complex, delphinidin can make hydrogen bonds with the side chain of Gln161 and the backbone carbonyl group of Met249 in the SH2 domain, and with the backbone carbonyl group of Tyr343 in the catalytic kinase domain. Delphinidin can also interact hydrophobically with Ile402 and Met344 of the kinase domain. The interaction between the SH2 domain and delphinidin could influence protein–protein interactions. The interaction with the kinase domain could induce structural distortion of ATP or the substrate binding site, and thus reduce the catalytic activity of Fyn kinase allosterically (Fig. 8B). Thus, delphinidin inhibited Fyn kinase activity by direct binding. Therefore, delphinidin inhibited the phosphorylation of TNF-α-induced JNK, p38, Akt, p90RSK, MSK1, and ERK. Consequently, delphinidin inhibited the activation of TNF-α-induced AP-1 and NF-κB, and COX-2 expression.

C. EGCG–ZAP-70, Fyn

EGCG is a major polyphenolic catechin in tea, and exerts cancer preventive activity at a variety of organ sites (102), including skin, lung, oral cavity, esophagus, stomach, small intestine, colon, pancreas, and mammary gland (227, 228, 233).

1. ZAP-70. The ZAP-70 is a ζ chain-associated 70-kDa protein of tyrosine kinase and plays a critical role in T cell receptor-mediated signal transduction and the immune response (30, 31). A high level of ZAP-70 expression is observed in leukemia, which suggests ZAP-70 as a logical target for immunomodulatory therapies (93). EGCG has been reported to regulate cluster of differentiation 3 (CD3)-mediated T cell receptor signaling in leukemia through the inhibition of ZAP-70 kinase (188). EGCG was found to bind with ZAP-70 *ex vivo* and *in vitro* with a binding affinity ($Kd = 0.6207\,\mu M$). Molecular modeling data, supported by site-directed mutagenesis experiments, showed that EGCG could form a series of intermolecular hydrogen bonds and hydrophobic interactions within the ATP-binding site, which may contribute to the stability of the ZAP-70–EGCG complex. The hydroxyl groups of EGCG at the A-ring, similar to other known protein kinase inhibitors, appear to form two hydrogen bonds with the kinase hinge region at the backbone carbonyl group of Glu415 and the amide group of Ala 417 (Fig. 10). The A–C-ring system acts as an adenine mimic and likewise interacts with the front cleft hydrophobic pocket. The gallate moiety (D-ring) occupies mainly the hydrophilic and solvent-exposed pocket covered by the G-loop. The side chains of lysine (Lys369) and aspartate (Asp479) may form a network of hydrogen bonds with the hydroxyl groups of the B-ring. In addition, the side chain of Glu386 is expected to form a hydrogen bond with one of the hydroxyl groups of this ring. The mutant experiments, in which both Lys369 and Asp479 were substituted, further suggest that Lys369 and Asp479 are required for the essential interactions of ZAP-70 with EGCG within the catalytic site (Fig. 10). Thus, EGCG effectively suppressed ZAP-70 by direct binding, linker for the activation of T cells, phospholipase Cγ1, ERK, and MAPK kinase activities in CD3-activated T cell leukemia. Consequently, the activation of AP-1 and interleukin-2 (IL-2) induced by CD3 was dose-dependently inhibited by EGCG treatment.

2. Fyn. An *in vitro* protein-binding assay revealed that EGCG directly bound to the GST-Fyn-SH2 domain but not to the GST-Fyn-SH3 domain, and inhibited kinase activity of Fyn. The Kd value for EGCG binding to the Fyn SH2 domain was $0.367 \pm 0.122\,\mu M$ and *B*max was 1.35 ± 0.128 nmol/mg (76). Thus, EGCG could inhibit the phosphorylation of EGF-induced p38, activating transcription factor-2 (ATF-2) and STAT1 with attenuated cell transformation.

D. Quercetin–MEK1, PI3K

Quercetin is a flavonol ubiquitously present in plant-derived foods and medicines. Its cancer-preventive effects have been attributed to various mechanisms, including their antioxidative activity, the inhibition of enzymes that activate carcinogens, the modification of signal transduction pathways, and interactions with receptors and other proteins (142).

1. MEK1. Quercetin is found to directly bind with Raf1 and MEK1 *ex vivo* and *in vitro*. However, quercetin showed stronger inhibition in kinase activity of MEK1 than that in Raf1 (120). Interestingly, quercetin could be docked to the pocket separate from but adjacent to the ATP-binding site of MEK1 (Fig. 9B). The hydroxyl group at the 7 position can make a hydrogen bond with the backbone carbonyl group of Val127 in the ATP noncompetitive binding site. In addition, several van der Waals interactions exist with the hydrophobic surface formed by Ile99, Ile141, Phe209, and Leu118. The C-ring interacts with the residues in the activation loop of the inactive MEK1. Val211 and Leu215 form van der Waals interactions with the C-ring of quercetin. The hydroxyl group at the 3′ position of the C-ring can make a critical hydrogen bond with the backbone amide group of Ser212. These interactions of quercetin with the activation loop would lock MEK1 into a catalytically inactive species by stabilizing the inactive conformation of the activation loop. Thus, quercetin inhibited TPA-induced the phosphorylation of ERK and p90RSK, and the activation of AP-1 and NF-κB. Consequently, quercetin inhibited TPA-induced cell transformation (Fig. 9B).

It is noted that resveratrol and kaempferol showed weaker inhibition on MEK1 activity than quercetin. The docking data appear to explain the reason. Although the binding model of resveratrol appears similar to that of quercetin, the 3′ position of resveratrol is placed on the 3′ position of quercetin. However, the lack of the hydroxyl group at the 3′ position of resveratrol would result in the failure of the formation of the hydrogen bond between resveratrol and the backbone amide group of Ser212 (120). Kaempferol is a compound structurally related to quercetin. The structural difference between kaempferol and quercetin is only the lack of the hydroxyl group at the 3′ position, which results in the failure of the formation of the hy-

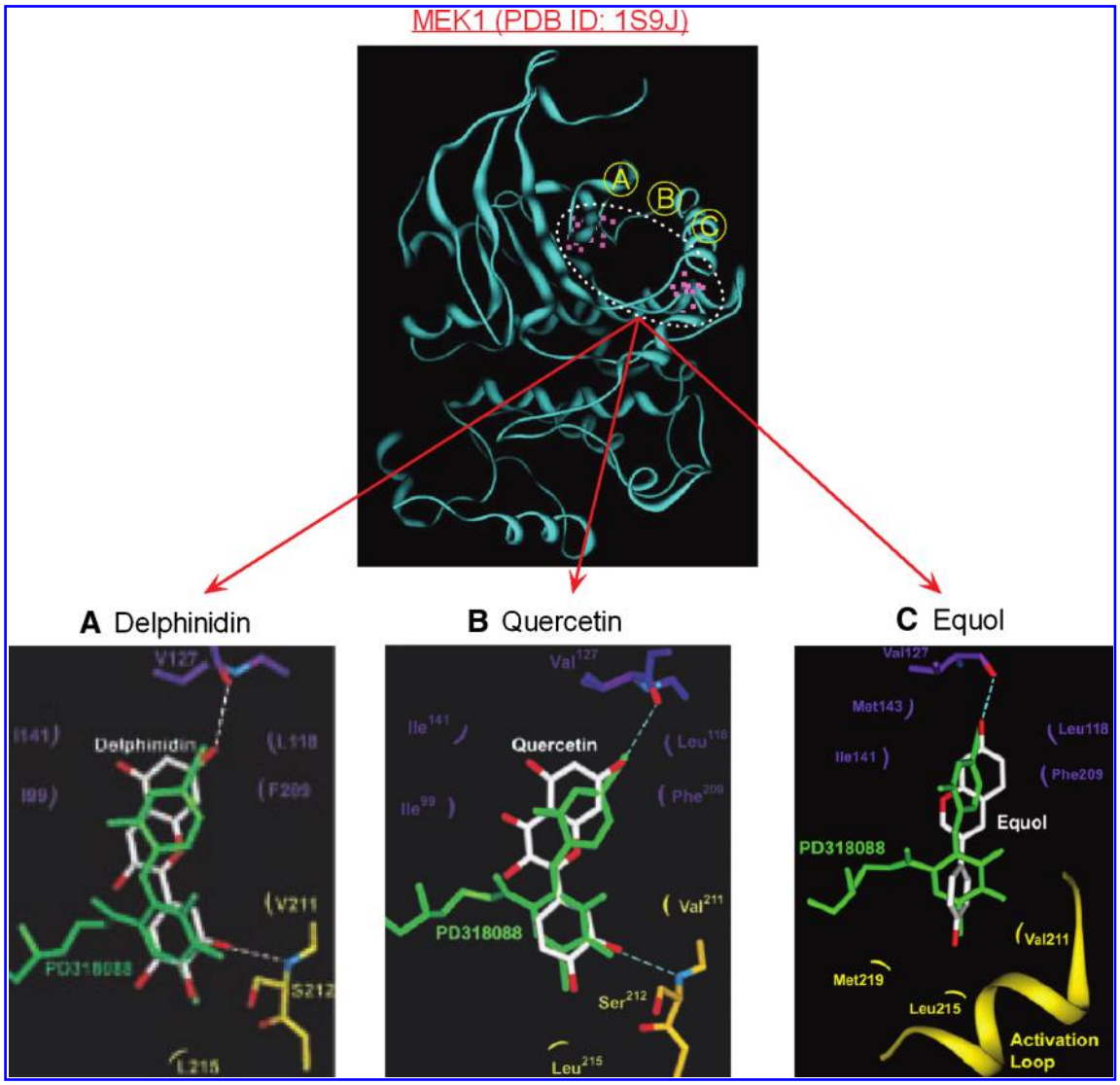

FIG. 9. Molecular modeling of delphinidin, quercetin, and equol binding to MEK1. (*Top*) The tube model of MEK1 structure from PDB (ID: 1S9J). *Pink points* show the direct binding sites of flavonoids with MEK1. *White ellipse* represents the binding region of these flavonoids with MEK1. (**A**) Delphinidin–MEK1 docking. *White line*: delphinidin; *Green line*: PD318088; *Purple line*: backbone of MEK1; *Yellow line*: activation loop site of MEK1; *Arc line*: *van der waals* interaction of delphinidin to MEK1. Hydrogen bonds are indicated by *white line* (96). (**B**) Quercetin–MEK1 docking. *White line*: quercetin; *Green line*: PD318088; *Purple line*: backbone of MEK1; *Yellow line*: activation loop site of MEK1; *Arc line*: *van der waals* interaction of quercetin to MEK1. Hydrogen bonds are indicated by *blue line* (120). (**C**) Equol docking. *White line*: equol; *Green line*: PD318088; *Purple line*: backbone of MEK1; *Yellow line*: activation loop site of MEK1; *Arc line*: *van der waals* interactions of equol to MEK1. Hydrogen bonds are indicated by *blue line* (98). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

drogen bond between kaempferol and the backbone amide group of Ser212 as the same reason as resveratrol (120).

2. PI3K. The X-ray crystallographic structure of PI3Kγ bound to quercetin revealed that quercetin fits into the ATP-binding site with a Kd value of 0.28 μM, which is slightly higher than that of myricetin (211). The PI3K complex has a shift in the quercetin so that the chromone appears to make three hydrogen bonds with the backbone of residues 880–882 in the linker region (208). Two of these hydrogen bonds are formed with the chromone 3- and 5-hydroxyls. Additionally, the ketone moiety interacts with the amide of Val-882 in a manner analogous to the N1 of the ATP adenine. Cell signaling investigation showed that the direct binding of PI3K by quercetin inhibited PI3K kinase activity and downregulated Akt signaling, including inhibition of AP-1 and NF-κB activation with attenuated matrix metalloproteinase-9 (MMP-9) expression and cell migration (89).

E. Caffeic acid–Fyn

Caffeic acid is a major dietary hydroxycinnamic acid and is derived from fruits, vegetables, grains, and coffee. Caffeic acid has been reported to have many bioactivities although

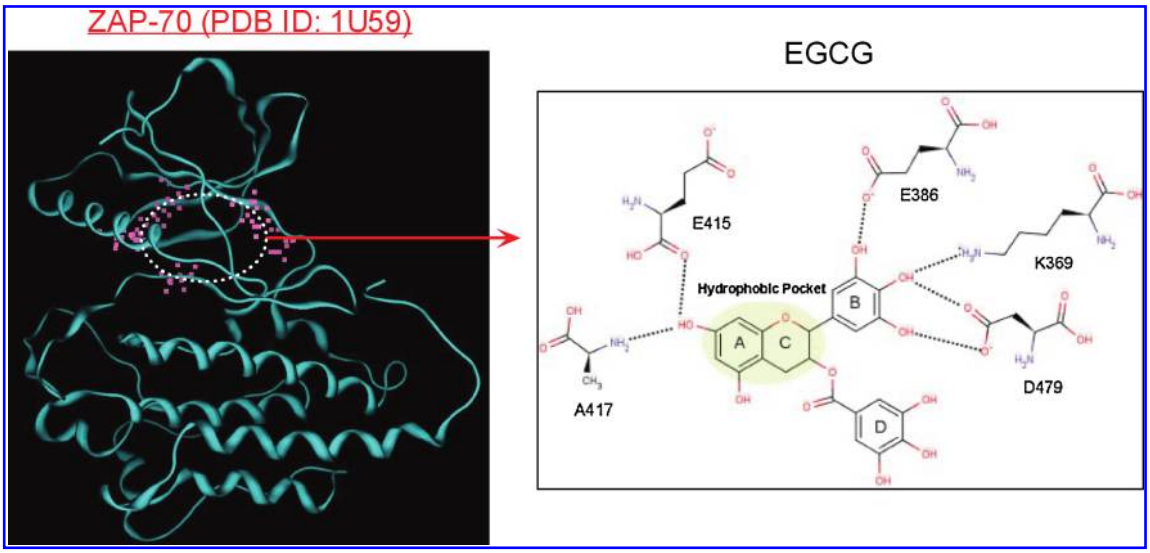

FIG. 10. Molecular modeling of ZAP-70 binding to EGCG. (*Left*) The tube model of ZAP-70 structure from PDB (ID: 1U59). *Pink points* show the directly binding sites of EGCG with ZAP-70. *White ellipse* represents the binding region of EGCG with ZAP-70. (*Right*) EGCG–ZAP–70 docking. *Yellow ellipse:* the hydrophobic pocket; *Dotted line*: hydrogen bonds (188). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

caffeic acid is a nonflavonoid phenolic compound. Habitual coffee consumption has been associated with a substantially lower risk of mortality as well as degenerative, progressive, and chronic diseases, including Alzheimer's disease, Parkinson's disease, type 2 diabetes, and coronary heart disease (77, 147).

Pull-down assays revealed that caffeic acid directly bound to Fyn noncompetitively with ATP (99). Modeling data showed that caffeic acid is docked to the putative allosteric site, rather than the ATP-binding site (92). Caffeic acid can make a salt bridge with Arg160 of the SH2 domain and forms hydrogen bonds with the backbone atoms of Tyr343 and Asn345 in the hinge region of the kinase domain (Fig. 8A). In addition, some hydrophobic interactions with Met249, I402, and M344 would also be possible. The interaction between the SH2 domain and caffeic acid could have some influence on protein–protein interaction through the SH2 domain. The interaction with the hinge region could also induce some structural distortion of the ATP-binding site; thus, reduce the binding affinity of ATP and the catalytic activity of the kinase domain allosterically (Fig. 8A). Cell signaling investigations showed that the direct binding of Fyn by caffeic acid suppressed Fyn kinase activity and downregulated MAPKs signaling, COX-2 expression through the inhibition of UVB-activated AP-1 and NF-κB activity in mice (99).

F. Equol–MEK1

Equol is a metabolite of daidzein, which is a major isoflavone of soybean (15). Soybean is an excellent source of dietary phenolic substances in the Asian diet. In many cell types, equol has higher activity than daidzein in terms of its antioxidant properties and anticancer activity (98, 231). Pull-down assays showed that equol directly bound to MEK1 without competing ATP (98). Modeling data further suggest that equol is docked to a pocket separate from but adjacent to the ATP-binding site similar to PD318088 (ATP noncompetitive inhibitor of MEK1) as observed in the crystal structure of the MEK1–PD318088 complex (Fig. 9C). The hydroxyl group at the 7 position of equol can make a hydrogen bond with the backbone carbonyl group of Val127 in the ATP noncompetitive binding site. In addition, several van der Waals interactions occur with the hydrophobic surface formed by Ile141, Met143, Phe209, Val211, and Leu118. The benzyl ring moiety of equol would interact with the residues in the activation loop of the nonphosphorylated MEK1. Val211, Leu215, and Met219 can form van der Waals interactions with the benzyl ring of equol. The hydroxyl groups at the 4′ position can not form any hydrogen bonds with the activation loop in the model structure of MEK1. The putative interactions between equol and the activation loop would lock MEK1 into a catalytically inactive formation by stabilizing the inactive conformation of the activation loop (98).

Interestingly, daizein, having additional carbonyl group at the 4 position, compared with equol is not docked to the adjacent to the ATP-binding site of MEK1 as equol because the carbonyl group of daidzein collides with the hydrophobic surface formed by Phe209, Val211, and Leu118. It may be the reason why equol had stronger inhibition on MEK1 kinase activity, the phosphorylation of downstream ERK and p90RSK, and cell transformation than daizein (98).

G. RSVL–MEK1

RSVL, a phytoalexin polyphenol present in grapes and red wines, has been shown to exert chemopreventive effects against cancer based on its inhibition of cellular events associated with tumor initiation, promotion and progression in both cell culture and animal models (14, 115, 183). RSVL2 was at least 10-fold more effective than RSVL in preventing EGF-

induced cell transformation with less toxicity (185). Pull-down assays showed that RSVL2 directly bound to MEK1 and did not compete with ATP (122). GST-MEK1 1-206, containing the N-terminal and binding region of PD184352 (ATP noncompetitive inhibitor of MEK1), interacted strongly with RSVL2, whereas GST-MEK1 1-67, which contains only the N-terminal, did not interact. Thus, RSVL2 is a potent inhibitor of MEK1 without competing with ATP, and the binding region of RSVL2 with MEK1 appears similar to that of the MEK1 inhibitor PD184352. Modeling data suggest that RSVL2 is docked to a pocket separate from, but adjacent to the ATP-binding site, in a manner similar to PD318088 as demonstrated in the crystal structure of the MEK1–PD318088 complex. The hydroxyl groups at the A-ring of RSVL2 can form a hydrogen bond with the backbone carbonyl group of Val127 in the ATP noncompetitive binding site. In addition, van der Waals interactions exist with the hydrophobic surface formed by Ile99, Ile114, Met143, Val127, Phe209, and Leu115. In particular, Phe209 interacts with the A-ring of RSVL2 in an edge-to-face way similar to the interaction of PD318088 with MEK1. The B-ring interacts with the residues in the activation loop of the nonphosphorylated MEK1. Val211 and Leu215 form van der Waals interactions with the B-ring of the inhibitor. One of the hydroxyl groups at the meta position of the B-ring forms a critical hydrogen bond with the backbone amide group of Ser212. The interactions of RSVL2 with the activation loop appear to lock MEK1 into a catalytically inert enzyme by stabilizing the inactive conformation of the activation loop. Notably, the only structural difference between RSVL2 and resveratrol is the additional 2 hydroxyl groups at the meta positions of the B-ring. The lower inhibitory activity of resveratrol may be due to the lack of the hydroxyl group at the meta position of the B-ring. The absence of this hydroxyl group can prevent resveratrol to form a hydrogen bond with the backbone amide group of Ser212, which is the key interaction for stabilizing the inactive conformation of the activation loop. Additional cell signaling data further supported that the direct binding of MEK1 by RSVL2 downregulated downstream molecular events including TPA-induced the phosphorylation of MEK1, ERK and p90RSK, AP-1 activation, and cell transformation (122).

H. Procyanidins–MEK1, MKK4

Procyanidin B2 is a polyphenolic compound, widespread in nature and in processed foodstuffs such as cocoa, chocolate, red wine, and fruit juice. Cocoa exhibits higher antioxidant activity than red wine, green tea, and black tea and exerts beneficial effects on cardiovascular diseases, some types of cancers, and Aβ-induced neurotoxicity (215). Several lines of studies suggest that procyanidin B2 can exert several physiological effects, such as antioxidant activity, antitumor effects, and protection against DNA damage induced by Fe(II)/H_2O_2 (38).

1. MEK1. Pull down assays demonstrated that procyanidin B2 directly bound with MEK1 without competing with ATP. A kinase assay revealed that procyanidin B2 inhibited the kinase activity of MEK1. Therefore, TPA-induced MEK1/ERK/p90RSK signaling was inhibited by procyanidin B2 with attendant inhibition of AP-1 and NF-κB transcriptional activities, and COX-2 expression and cell transformation (97).

2. MKK4. Pull down assays revealed that procyanidin B2 directly bound to MKK4 and inhibited MKK4 activation to attenuate 4-hydroxynonenal-induced apoptosis of PC12 cells (38).

IV. Signaling Pathways Regulated by Direct Binding of Flavonoid–Protein Kinase

Cellular signal pathways play important roles in the control of many fundamental cellular processes (150, 176). These pathways trigger a wide variety of cellular events such as increasing expression of detoxifying enzymes and/or antioxidant enzymes, inhibiting cell cycle progression and cell proliferation, inducing differentiation and apoptosis, inhibiting expression and functional activation of oncogenes, increasing expression of tumor-suppressor genes, and inhibiting angiogenesis and metastasis by modulating cellular signaling pathways (36,197). Most of these signal pathways are regulated by protein kinases. Therefore, protein kinases are now recognized as potential molecular targets for chemoprevention by flavonoids (87). Flavonoids have been found to have cancer chemoprevention by modulating some cellular signal pathways. The direct binding of flavonoids to these protein kinases gives direct evidence how flavonoids modulate these cellular signaling pathways. According to the information from recent publications, the following signaling pathways are at least regulated through the direct binding of protein kinases by flavonoids (Fig. 11).

A. PI3K–Akt signaling

The PI3K–Akt pathway plays a critical role in mammalian cell survival signaling and has been shown to be activated in various cancers (12, 33, 40). PI3Ks belong to a conserved family of lipid kinases that phosphorylate the 3′-hydroxyl group of phosphoinositides. The PI3K superfamily is grouped into three classes: I, II, and III (52). Furthermore, two subclass PI3K (class IA and class IB) are included in class I PI3K, and the most important PI3K proteins are those that belong to class IA in regulating proliferation and tumorigenesis (16). PI3Ks have a role in cancer. Increased levels of PI3K products have been seen in colorectal tumors (158) and in breast cancers (70). Dephosphorylation of PI3K products by the lipid-phosphatase activity of PTEN suppresses tumor formation (18, 26, 223). The PI3K inhibitors wortmannin and LY294002 have been crucial in deciphering the roles of PI3Ks in cellular processes.

The most well-characterized product of PI3K reaction is phosphatidylinositol-3,4,5-trisphosphate (PIP3), a critical second messenger that recruits Akt for activation of multiple cellular functions (232). It has been known that Akt is activated by phospholipids binding and phosphorylation at Thr308 by pyruvate dehydrogenase kinase 1 (PDK1) or at Ser473 by ToRC2 (10). Akt is a serine/threonine kinase, which comprises three highly homologous known as Akt1, Akt2, and Akt3. They are products of distinct genes, but exhibit greater than 80% homology at the amino acid level. Each isoform possesses an N-terminal pleckstrin homology (PH) domain of approximately 100 amino acids. The PH domain is followed with the kinase catalytic and regulatory domains (146). Activated Akt functions to promote cell survival by inhibiting apoptosis through inactivation of several

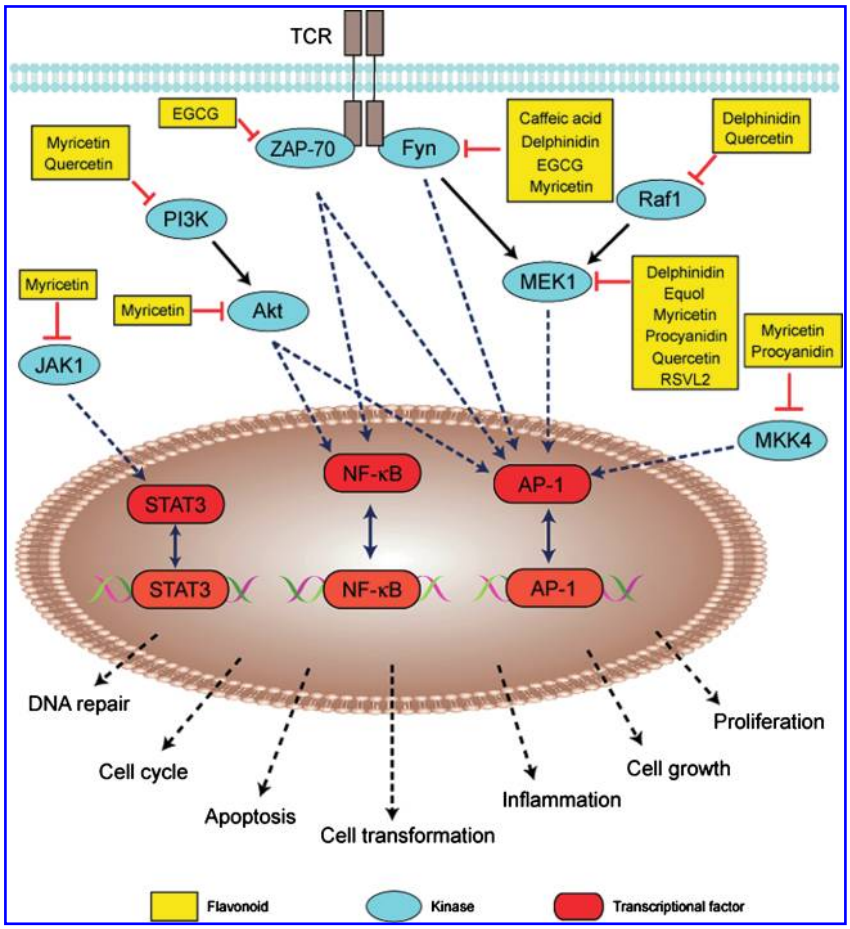

FIG. 11. Cellular signaling pathways regulated by the direct binding of protein kinases with flavonoids. Flavonoids are indicated within *square mark*. Kinases are indicated within *ellipse mark*. Transcript factors are indicated within *ellipse–square mark*. *Dotted arrows* represent the indirect downstream events. Solid *arrows* represent the direct binding of flavonoids to protein kinases. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

pro-apoptotic factors, including Bcl-2-associated death promoter (Bad), Forkhead transcription factors, and caspase-9 (24, 27, 171). Studies have also shown that Akt regulates the NF-κB pathway via phosphorylation and activation of the molecules in the NF-κB signaling pathway (152, 170). Akt also regulates AP-1 activation through phosphorylation of the molecules in the MAPK signaling pathway. Thus, the PI3K–Akt pathway is also considered to be an attractive target for cancer prevention or treatment (126). Indeed, many small molecules as Akt-specific inhibitors were investigated and identified (114). Myricetin and quercetin have been found to directly bind to the ATP-binding site of both PI3K (211) and Akt (113) to alter PI3K–Akt-mediated signaling pathways and downstream events, such as cell transformation inhibition (Fig. 11).

B. Raf–MEK1–MAPK signaling

The MAPK pathway consists of a three-tiered kinase core where a MAP3K (*e.g.*, Raf) activates a MAP2K (*e.g.*, MEK1/2) that activates a MAPKs (*e.g.*, ERK, JNK, and p38), resulting in the activation of NF-κB and AP-1 with cell growth and cell survival (180, 182). Raf is a Ser/Thr kinase and is normally activated by multiple events (34, 53, 60, 127, 226, 229). The mammalian Raf gene family consists of A-Raf, B-Raf, and Raf-1 (C-Raf). All three Raf family members are able to phosphorylate and activate MEK, but the different biochemical potencies have been observed (B-Raf > Raf-1 >> A-Raf) (11). MEK is a Tyr and Ser/Thr dual specificity protein kinase, and its activity is positively regulated by Raf phosphorylation on serine residues in the catalytic domain. ERK1 and 2 are Ser/Thr kinases and their activities are positively regulated by phosphorylation mediated by MEK1 and MEK2 (134). It has been reported that MAPKs are activated in several types of cancer and that the activation of MAPKs is also linked to cancer angiogenesis, invasion, and metastasis (61). Therefore, the molecules in the MAPK pathway have also received increasing attention as target molecules for cancer prevention and therapy (134). In fact, inhibitors of Raf (*e.g.*, L-779450, ZM336372, and Bay43-9006) and MEK (*e.g.*, PD98059, U0126, PD184352, PD0325901, and array MEK inhibitors) and some downstream targets have been developed, some of which are used currently in clinical trials for cancer therapy (73, 128, 133, 134, 186).

Regarding flavonoids, it has been reported that caffeic acid (99) delphinidin (88), EGCG (76), and myricetin (95) bind directly with Fyn. Delphinidin (96) and quercetin (120) bind directly with Raf1. Moreover, delphinidin (96), equol (98), myricetin (121), RSVL2 (122), and quercetin (120) bind directly with MEK1 (Fig. 11). Myricetin (103) and procyanidin (38) bind directly with MKK4. Additional cell signaling data further supported that the direct binding of these protein kinases by flavonoids downregulated downstream molecular events including TPA-induced the phosphoryla-

tion of MEK1, ERK, and p90RSK, AP-1 activation, and cell transformation.

C. JAK–STAT3 signaling

The JAK-STAT pathway is one of the important signaling pathways downstream of cytokine receptors, which signaling regulates a variety of important cellular functions (90). JAK family is one of ten recognized families of nonreceptor tyrosine kinases. Four members of this family, including JAK1, JAK2, JAK3, and tyrosine kinase 2 (Tyk2), are found in mammals. Seven JAK homology (JH) domains are identified and numbered from carboxyl to the amino terminus. Both tyrosine kinase domain (JH1) and pseudo-kinase domain (JH2) are located at its carboxyl termini (155, 162, 225). The tyrosine phosphorylation of STAT3 is mediated by the phosphorylation of JAK kinase (204).

STATs are DNA-binding transcriptional factors. To date, STAT proteins were identified seven proteins in mammals, such as STAT1, 2, 3, 4, 5A, 5B, and 6. The C-terminal portion of STATs contains several key elements required for STAT activation and function, including in Src-homology 2 (SH2) domain, and transactivation domain (190, 216, 217). In the N-terminal portion, STATs contain the DNA-binding domain (80, 224). There is a high frequency of activation of Stat1, Stat3, and Stat5 in human cancers, with higher incidence of abnormal Stat3 activation in almost all the tumors studied (21, 23, 204). STAT3 have been reported to be constitutively activated in a number of human malignancies including breast cancer, lung cancer, head and neck cancer, brain tumors, melanoma, multiple myeloma, lymphomas, acute myelocytic leukemia (CLL), chronic lymphocytic leukemia (AML), large granular lymphocyte (LGL), ovarian cancer, prostate cancer, renal cell carcinoma, and pancreatic adenocarcinoma (107). Therefore, the JAK1–STAT3 pathway has been suggested to play a critical role in cell transformation and carcinogenesis. We found that myricetin directly bound with JAK1, and inhibited EGF-induced STAT3 phosphorylation, DNA binding, and cell transformation (112) (Fig. 11).

Flavonoids might exert modulatory effects in cells through selective actions at different components of a number of protein kinases, which may be independent of classical antioxidant capacity. Oxidative stress has a diverse effect on signaling pathways, particularly the MAP kinase cascade (201, 205). There is strong evidence linking the activation of JNK to neuronal loss in response to a wide array of pro-apoptotic stimuli in both developmental and degenerative death signaling (28, 49). In the context of oxidative insults in neurons, JNK is activated by 4-HNE (25, 192), through reduced expression of SOD1(130), by hydrogen peroxide (44), and by oxLDL (178). A number of flavonoids have been reported to inhibit this activation (219). In addition to MAP kinase, the modulation of signaling through Akt (47, 41, 45) may also be important in neuronal survival. The activation of Akt in some neurons has been shown to lead to an inhibition of proteins linking to the cell death machinery, such as the proapoptotic BAD (234) and caspases (20, 46), thus promoting cell survival. BAD itself is regulated by phosphorylation of two serine residues, Ser112 and Ser136 (234), of which, the Ser136 site can be specifically phosphorylated by Akt (46, 51). Thus, flavonoids acting these cascades will protect neuronal survival against oxidative stress.

Although selective inhibitory actions at these kinase cascades may be beneficial in cancer, proliferative diseases, inflammation, and neurodegeneration, they could be detremental during development, particularly in the immature nervous system, when protein kinase signaling regulates survival, synaptogenesis, and neurite outgrowth (219). In post mitotic neurons, MAP kinase and PI3K cascades have been reported to regulate synaptic plasticity and memory formation (154, 123, 198). Thus, flavonoid interactions with these pathways could have unpredictable outcomes and will be dependent on both the cell type and the disease studied.

V. Binding Sites and Protein Kinase Selectivity of Flavonoids

A. The sites of flavonoids binding to protein kinases

Protein kinase inhibitors discovered to date have been found to bind with different sites of protein kinase, which involve ATP-binding site, activation loop, and allosteric site. Figure 12 shows a typical example of the binding sites of MEK1 inhibitors. ATP is located in ATP-binding site by six hydrogen bonds with the hinge region amino acids Glu144, Met146, Ser150, Lys97, Lys192, and Ser194 of MEK1 (Fig.12A). K252a, an ATP-competitive inhibitor (66), makes two hydrogen bonds with the hinge region amino acids Glu144 and Met146 of MEK1 (Fig.12B). Inhibitor G8935 binds to the activation loop by hydrogen bonds with Asp208, Val211, and Ser212 of MEK1 (Fig.12C). U0126 (66) binds to the allosteric site by hydrogen bonds with Lys97, Asp208, Phe209, and Val211 of MEK1 (Fig.12D).

The information for the sites of flavonoids binding to protein kinases is summarized in Table 1 according to up-dated reports. Flavonoids can directly bind to protein kinases at ATP-binding site, activation loop as well as allosteric site of protein kinases.

1. ATP-binding site. ATP binds in the cleft with the adenine ring forming hydrogen bonds with the protein kinase 'hinge', which is the segment connecting the amino- and carboxy-terminal kinase domains. The ribose and triphosphate groups of ATP bind in a hydrophilic channel extending to the substrate binding site that features conserved residues that are essential to catalysis (56, 137, 202). Despite the fact that the ATP-binding site is highly conserved among the protein kinases (59, 19, 105), the architecture in the regions proximal to the ATP-binding site does afford some key diversity. The binding site of ATP in protein kinases involves five regions: (a) adenine region, (b) sugar region, (c) hydrophobic pocket (or selectivity pocket), (d) hydrophobic channel, and (e) phosphate binding region (59). The hydrophobic pocket plays an important role for inhibitor selectivity. Its size is typically controlled by two amino acid residues (*e.g.*, at positions 120 and 183 of PKA) (45). Thus, the hydrophobic pocket is exploited by most of the protein kinase inhibitors, but is not used by ATP.

The inhibitor that binds to the ATP binding site in an ATP-mimetic manner is referred to type I inhibitor (235). Most ATP competitive inhibitors are addressing one to three hydrogen bonds to the amino acids located in the hinge region of the target protein kinase, thereby mimicking the hydrogen bonds that are normally formed by the adenine ring of ATP (125,

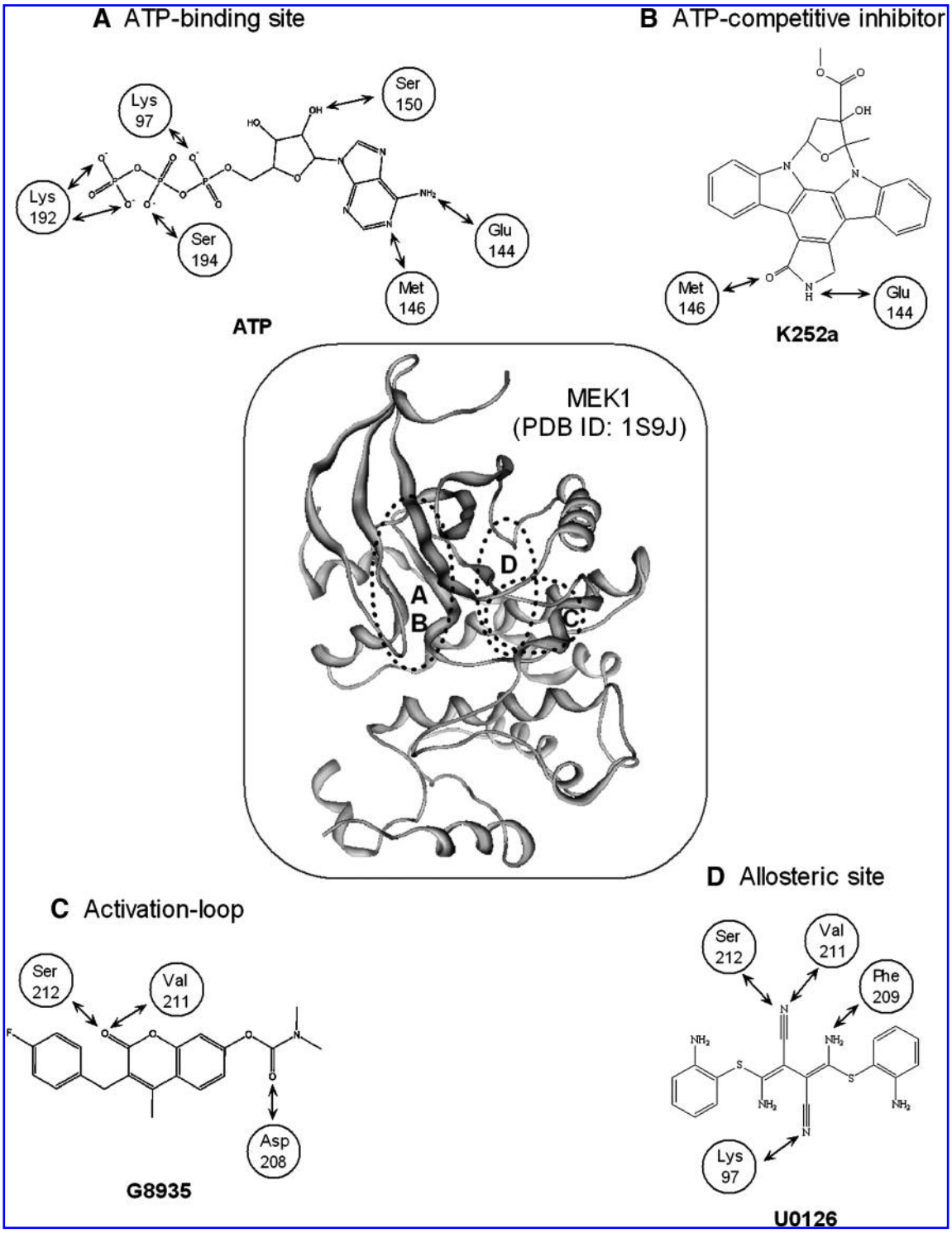

FIG. 12. Binding sites of MEK1 inhibitors. The three-dimensional structure of MEK1 is obtained from PDB (ID: 1S9J) (*Central*). The binding sites and chemical structure of MEK1 inhibitors show (**A**) ATP-biding site bound with ATP; (**B**) ATP-competitive inhibitor, K252a (66); (**C**) Activation-loop bound with G8935 (66); (**D**) Allosteric site bound with U0126 (74). The amino acids of MEK1 involved in H-bonding with flavonoids are *encircled*.

202). Figure 12A shows the binding site of ATP and K252a in MEK1, an ATP-competitive inhibitor (66).

With regard to flavonoids binding to the ATP pocket, the most plausible current hypothesis is that the chromenone moiety of the flavonoid acts as a mimetic of the adenine moiety of ATP. The number and substitution of hydroxyl groups on the B-ring and the degree of unsaturation of the C2–C3 bond appear important determinants of this particular bioactivity. The property of the hydrophobic pocket of the ATP-binding site as mentioned above appears the reason that

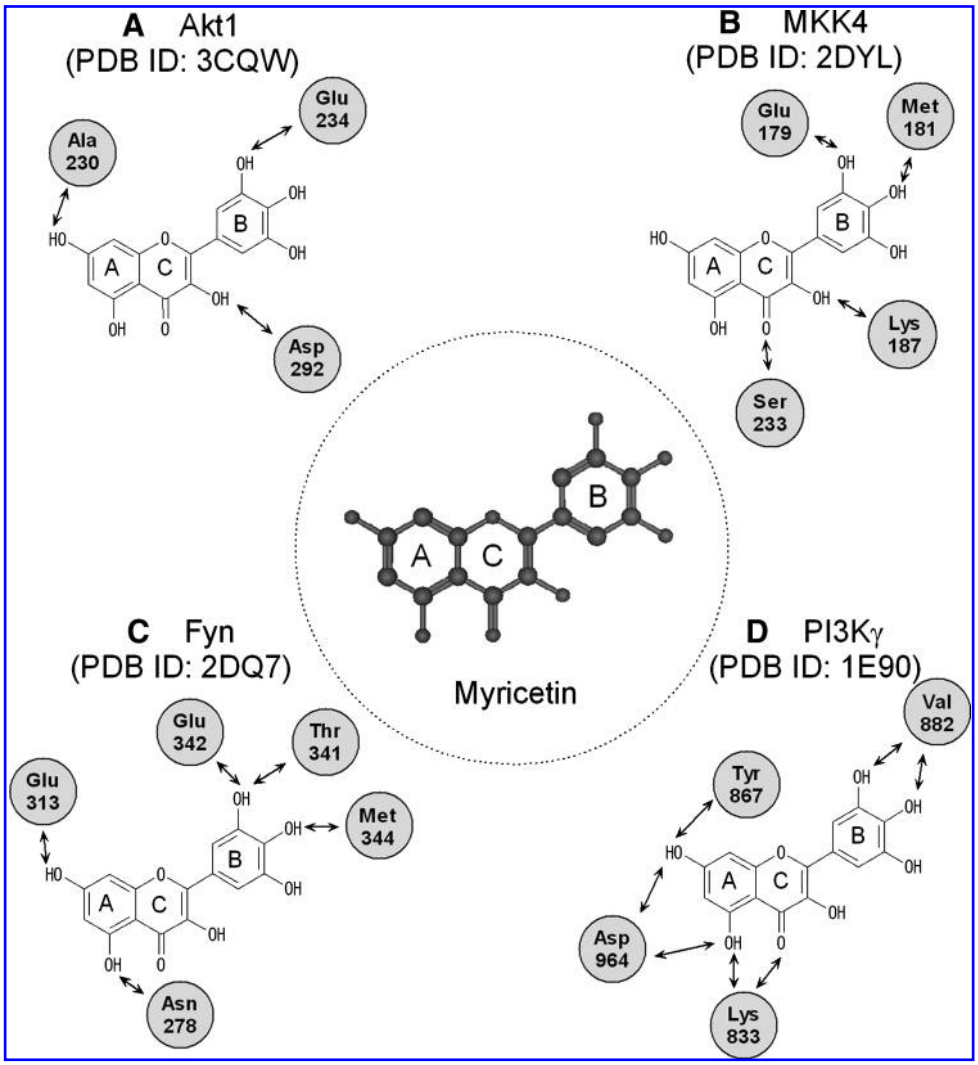

FIG. 13. Myricetin binds the ATP-pocket of multiple protein kinases. The chemical structure of myricetin is shown in the middle. (**A**) Myricetin binds to the ATP-pocket of Akt. The three-dimensional structure of Akt is obtained from PDB (ID: 3CQW). Hydrogen bonds between myricetin and Akt1 are indicated by *arrows* (113). (**B**) Myricetin binds to the ATP-pocket of MKK4. The three-dimensional structure of MKK4 is obtained from PDB (ID: 2DYL). Hydrogen bonds between myricetin and MKK4 are indicated by *arrows* (103). (**C**) Myricetin binds to the ATP-pocket of Fyn. The three-dimensional structure of Fyn is obtained from PDB (ID: 2DQ7). Hydrogen bonds between myricetin and Fyn are indicated by *arrows* (95). (**D**) Myricetin binds to the ATP-pocket of PI3Kγ. The three-dimensional structure of PI3Kγ is obtained from PDB (ID: 2DQ7). Hydrogen bonds between myricetin and PI3Kγ are indicated by *arrows* (211).

different protein kinases can be selectively bound by some flavonoids. Moreover, the architecture in the regions proximal to the ATP-binding site also can afford some key diversity. Myricetin is a case in point, which has been found to directly bind to the ATP-pocket of Akt1 (113) with hydrogen bonds of Ala230, Glu234, and Asp292, MKK4 (103) with hydrogen bonds of Glu179, Met181, Lys187, and Ser233, Fyn (95) with hydrogen bonds of Asn278, Glu313, Glu342, Thr341, and Met344, and PI3Kγ (211) with hydrogen bonds of Lys833, Val882, Tyr867, and Asp964 in ATP-mimetic manner (Fig. 13).

2. Activation loop. The activation loop is conserved in all protein kinases, and is important in regulating protein kinase activity (2). The activation loop is marked by conserved DFG and APE motifs (which refer to one-letter amino acid abbreviations) at the start and end of the loop, respectively. Analysis of multiple protein kinase inhibitor co-crystal structures has revealed that all share a similar pharmacophore and exploit a conserved set of hydrogen bonds to the activation loop (144). The inhibitor-stabilized conformational rearrangement of the activation loop demonstrates that the active site of protein kinase is highly flexible and can be remodeled to accommodate a variety of inhibitors (125). This type of inhibitors is referred to type II inhibitor (235). Imatinib, an inhibitor for ABL1, KIT, and PDGFR (144), and sorafenib, an inhibitor for KIT, PDGFR, and Raf (212), are found to bind at the activation loop. MEK1 inhibitors including G8935 (74) (Fig.12C), PD098059, U0126, PD184352, and PD318088 (149) also bind at the activation loop of MEK1 without ATP competition (63).

As a typical case of flavonoids, the C-ring of quercetin interacts with the residues in the activation loop of the inactive MEK1. Val211, and Leu215 form van der Waals interactions with the C-ring of quercetin. The hydroxyl group at the 3′ position of the C ring can make a critical hydrogen bond with the backbone amide group of Ser212. These interactions of quercetin with the activation loop would lock MEK1 into a catalytically inactive species by stabilizing the inactive conformation of the activation loop. The predicted binding mode of quercetin is similar to that of PD318088. Interestingly, kaempferol and resveratrol, two compounds structurally related to quercetin, have a lesser inhibitory effect on MEK1, suggesting that the hydroxyl group at the 3′ position is important to interact with MEK1 (120).

3. Allosteric site. The allosteric site is outside the ATP-binding site. This type of inhibitor, referred as allosteric inhibitor, modulates protein kinase activity in an allosteric

manner, and tends to exhibit the highest degree of protein kinase selectivity because they exploit binding sites and regulatory mechanisms that are unique to a particular protein kinase (118). Allosteric regulation of protein function is a fascinating and wide-ranging means of controlling protein function. It encompasses a variety of triggers and an assortment of mechanisms for relaying the signal from the allosteric to the active site. The mechanisms can involve the opening or closing of the active site, modification of the conformation of the residues in the active site, changes to the rigidity or electrostatic properties of the active site, alteration of the dynamic properties of the protein as a whole or via a population shift in the protein's conformational ensemble. Indeed, allosteric effects may involve a combination of these mechanisms (118). In drug discovery it may provide an alternative means of targeting a protein by small-molecule drugs. The allosteric binding site may provide a better target than the active site as, being under less evolutionary pressure, may be more species specific (174, 179). This type of inhibitors includes MEK1 and MEK2 inhibitor CI-1040 (149) and U0126 (66, 74, 206), IKK inhibitor BMS-345541 (136), and Akt inhibitor Akt-I-1 (17, 124). The most well characterized allosteric kinase inhibitor is CI-1040, which inhibits MEK1 and MEK2 by occupying a pocket adjacent to the ATP-binding site (149). Figure 12D shows the chemical structure of U0126 and its hydrogen bonds with Lys97, Asp208, Phe209, and Val211 of MEK1 (66).

With regard to flavonoids, delphinidin has been reported to directly bind to Fyn kinase noncompetitively with ATP (88). Modeling data revealed that delphinidin was docked to the putative allosteric site where rosmarinic acid was expected to bind in the homology model structure of Fyn kinase (92). In the hypothetical structure of the Fyn kinase–delphinidin complex, delphinidin can make hydrogen bonds with the side chain of Gln161 and the backbone carbonyl group of Met249 in the SH2 domain, and with the backbone carbonyl group of Tyr343 in the catalytic kinase domain. Delphinidin appears also to interact hydrophobically with Ile402 and Met344 of the kinase domain. The interaction between the SH2 domain and delphinidin could influence protein-protein interactions.

Additionally, some small molecules form an irreversible covalent bond to the active site of protein kinase, most frequently by reacting with a nucleophilic cysteine residue. This type of inhibitor is referred to as covalent inhibitors (235). For example, the epidermal growth factor receptor (EGFR), HKI-272 (163), and CL-387785 (109) are developed to target a relatively rare cysteine residue located at the lip of the ATP-binding site (67). In our knowledge, there is no report on flavonoids as covalent inhibitors of protein kinases.

Several lines of studies have shown that one flavonoid can inhibit several protein kinases. The structure of flavonoids will determine whether they act as potent inhibitors of PKC, tyrosine kinase, and PI3K (9, 65). The number and substitution of hydroxyl groups on the B-ring and the degree of unsaturation of the C2–C3 bond appear important determinants of this particular bioactivity (9). Myricetin is a perfect example because myricetin is found to directly bind with JAK1 (112), PI3K (211), Akt (113), Fyn (95), MEK1 (121), and MKK4 (103) (Fig. 11).

Therefore, the potential of flavonoids binding to protein kinases will depend on both the structure of flavonoids (*e.g.*, the number and position of hydroxyl group on the B-ring, and the degree of unsaturation of the C2–C3 bond of flavonoids) and the properties of different protein kinase (*e.g.*, selectivity of ATP-pocket, the architecture in the adjacent to the ATP-binding site).

B. *Affinity of flavonoid binding to protein kinases*

Flavonoids have been suggested as rich protein kinase inhibitors, but are not yet a much explored source of potentially useful cancer chemopreventive and treatment drugs, although these compounds have a variety of physiological effects in animals. The target affinity and selectivity of flavonoid, compared with synthesized small compounds, are considered to be important points. Although there are not enough data to give a clear view about these properties, interesting data were obtained in the affinity comparison of PI3Kγ kinase ATP-binding site by plant-derived flavonoid quercetin and myricetin with the synthetic compound LY294002 (211). Quercetin and the closely related myricetin are plant-derived bioflavonoids, in particular, quercetin was used as the lead compound on which a variety of derivatives, including LY294002, were designed (208). LY294002 is a synthetic inhibitor for PI3K. All of three inhibitors are competitive inhibitors of ATP binding. The inhibitory activity (expressed as IC_{50}) of quercetin, myricetin, and LY294002 on PI3Kγ are reported previously as 3.8 μM, 1.8 μM, and 1.4 μM, respectively (Fig. 14). It is interested that their IC_{50} values appear to be correlated with their affinities for PI3Kγ because the Kd of quercetin, myricetin, and LY294002 for PI3Kγ are 0.28 μM, 0.17 μM, 0.21 μM, respectively (Fig.14). Surprisingly, the interaction of LY294002 with PI3K is quite different with that of quercetin as well as myricetin. The chromone moiety of quercetin occupies the space filled by the morpholino ring of LY294002. The 3′,4′-dihydroxyphenyl moiety at the 2 position of quercetin makes a putative hydrogen bond with NZ of Lys833 (211). The hydrogen bond between the 3′-OH of quercetin and the side chain of Lys833 mimics the interaction made by the ketone moiety of LY294002. This means that the chromone moiety of LY294002 is flipped 180° with respect to binding model in the quercetin-PI3K complex (Fig. 14). The flip of the LY294002 orientation relative to quercetin is possibly a consequence of the nonplanar morpholino group (211).

C. *Multiple targets* vs. *selectivity*

A highly selective drug can target a single molecule responsible for the etiology of a disease. However, most diseases involve multiple molecular abnormalities with more than one dysfunctional protein. Therefore, drugs whose efficacy is based on rebalancing the several proteins or events that contribute to the etiology, pathogenesis, and progression of a disease may be ideal (6). For example, sunitinib can target VEGF, platelet-derived growth factor (PDGF), KIT, and FMS-like tyrosine kinase 3 (FLT3) receptor tyrosine kinase. Sorafenib can target VEGF, PDGF, and Raf/MEK/ERK (138).

Alterations in multiple cellular signaling pathways are frequently found in many cancer cells. Perhaps this is the reason why the specific inhibitors that target only one pathway, most often failed in cancer treatment. The lack of selectivity of protein kinase inhibitor can sometimes be advantageous since

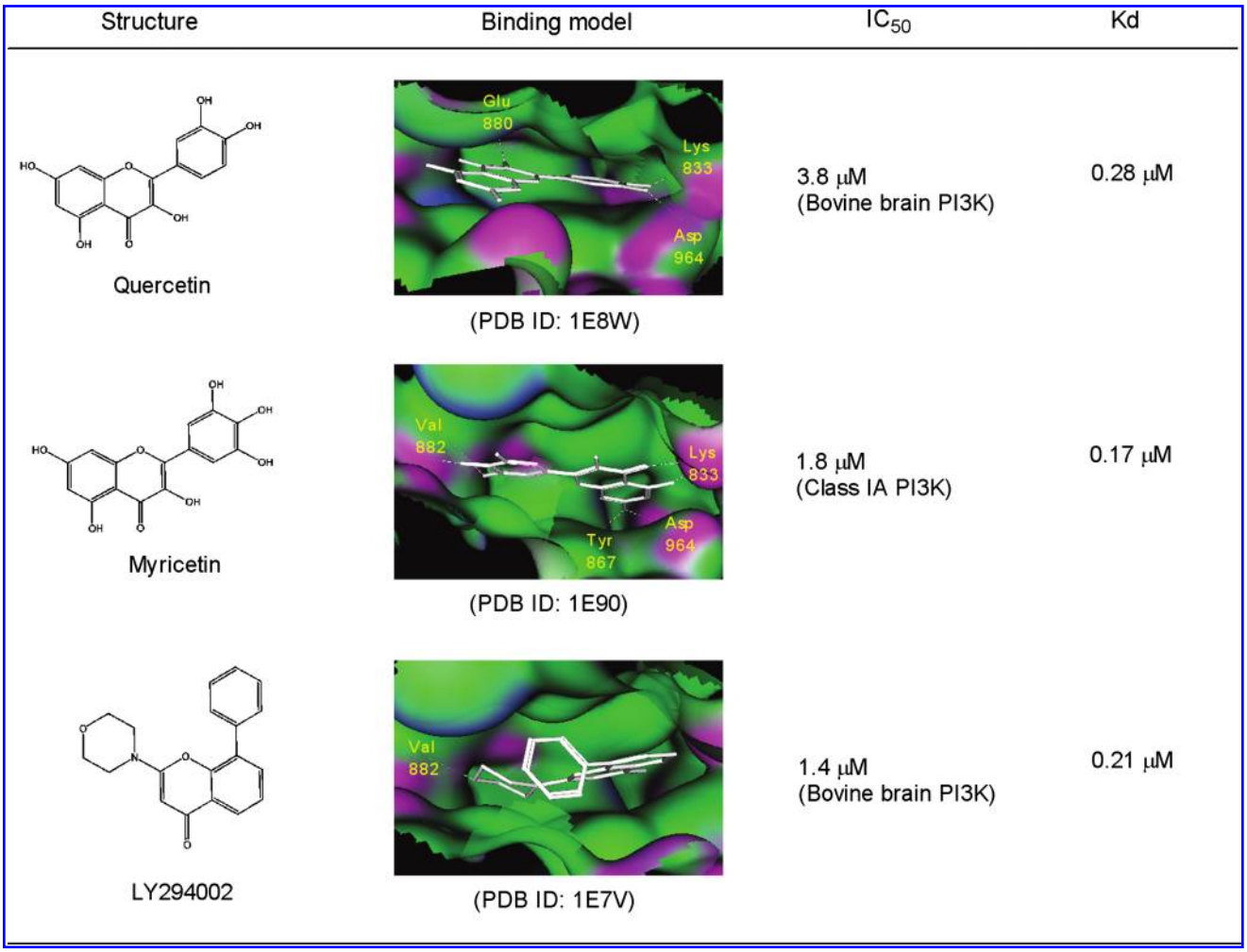

FIG. 14. The characteristics of quercetin, myricetin, and synthetic LY294002 binding to PI3K*η*. (*Left*) The chemical structure of quercetin, myricetin, and LY294002; (*Middle*) the molecular modeling of quercetin, myricetin, and LY294002 superposed on the ATP-binding site of PI3Kγ. The three-dimensional structure of the ATP-binding site of PI3Kγ superposed with quercetin, myricetin, and LY294002 are obtained from PDB (ID shown). (*Right*) The IC_{50} value and Kd constant of quercetin, myricetin, and LY294002 binding to PI3Kγ (211). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

anticancer drugs that act on multiple tyrosine kinases are thought to be more effective than those that are specific in their mode of action (156). Flavonoids that can suppress these multiple pathways would have great potential in cancer chemoprevention and cancer treatment. On the other hand, cross-reactivity with unrelated ATP-binding proteins is also a frequent problem, resulting in undesirable side effects that cause many protein kinase inhibitors to fail in either preclinical or clinical development (19). Therefore, multiple targets and side effect possibility of flavonoids as protein kinase inhibitors are required to be clarified further.

It has been reported that the molecular targets of cancer chemopreventive agents such as curcumin (5), resveratrol (3), guggulsterone (189), silymarin (7), and indole-3-carbinol (4) are similar to those currently being used to treat cancer. The evidence indicates that most of the plant-based agents used in traditional Ayurvedic and Chinese medicine contain a variety of flavonoids, and do indeed suppress multiple pathways (*e.g.*, those of NF-κB, AP-1, JNK, COX-2, cyclin D1, matrix metalloproteinase (MMP), inducible nitric oxide synthase (iNOS), human epidermal growth factor receptor 2 (HER2), EGFR, B-cell lymphoma 2 (bcl-2), B-cell lymphoma-extra large (bcl-XL), and TNF) (7, 8). Importantly, all of these signaling pathways have been found malfunctioning in cancer cells, resulting in cancer cell proliferation and inhibition of apoptosis (100, 108, 131, 181, 196). Therefore, it is important to design a strategy that could simultaneously target multiple cellular signaling pathways so that cancer cells could get killed effectively. Therefore, targeting multiple signaling by natural flavonoids has opened a new avenue for cancer chemoprevention and therapy. In fact, 48 of 65 drugs approved for cancer treatment between 1981 and 2002 were natural products, or based on natural products, or mimicked natural products in one form or another (145).

Although the role of protein kinases was focused on carcinogenesis in this review, most protein kinases play roles in a wide range of intracellular signaling that regulate cell proliferation, differentiation, apoptosis, inflammation, and aging

process (86, 197). Thus, flavonoids that directly bind these protein kinases will exert multiple physiological functions although they are required to be clarified in further studies. Moreover, the proteins directly targeted by flavonoids are not only limited to protein kinases to exert their physiological or pharmacological effects. For example, it has been reported that EGCG also can bind with vimentin and laminin to inhibit cell proliferation (57, 199). The data accumulation in flavonoids-interacted proteins will give a more whole view to explain why flavonoids exert a wide range of physiological functions in our body.

VI. Remarks and Perspectives

The data from *ex vivo* and *in vitro* experiments clearly indicate that some flavonoids including myricetin, quercetin, caffeic acid, daidzein (equol), delphinidin, EGCG, and resveratrol analogue (RSVL2) can directly bind to some central kinases such as PI3K, Akt, JAK1, Raf1, ZAP70, MEK1, MKK4, and Fyn, which regulate multiple cell signaling pathways in carcinogenesis processes. It is notable that one flavonoid can bind several protein kinases to target multiple pathways. Flavonoids with multiple targets will be ideal agents for treating some chronic disease such cancer because these diseases, especially cancer, always show alterations in multiple cellular signaling pathways. Therefore, regulation of multiple cell signaling pathways by flavonoids will control the behavior of cancer cells such as inhibition of cell growth and apoptosis induction which are regulated by multiple pathways. On the other hand, future investigations of the flavonoid–protein kinase complex using X-ray crystallography are required to elucidate the exact binding modes. Moreover, works on the interactions between protein kinases and various metabolites of flavonoids are also important to elucidate the exact molecules of flavonoids, which exert the bioactive function *in vivo.*

Acknowledgments

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References

1. Abd El Mohsen MM, Kuhnle G, Rechner AR, Schroeter H, Rose S, Jenner P, and Rice–Evans CA. Uptake and metabolism of epicatechin and its access to the brain after oral ingestion. *Free Radic Biol Med* 33: 1693–1702, 2002.
2. Adams JA. Activation loop phosphorylation and catalysis in protein kinases: Is there functional evidence for the autoinhibitor model? *Biochemistry* 42: 601–607, 2003.
3. Aggarwal BB, Bhardwaj A, Aggarwal RS, Seeram NP, Shishodia S, and Takada Y. Role of resveratrol in prevention and therapy of cancer: Preclinical and clinical studies. *Anticancer Res* 24: 2783–2840, 2004.
4. Aggarwal BB and Ichikawa H. Molecular targets and anticancer potential of indole-3-carbinol and its derivatives. *Cell Cycle* 4: 1201–1215, 2005.
5. Aggarwal BB, Kumar A, and Bharti AC. Anticancer potential of curcumin: Preclinical and clinical studies. *Anticancer Res* 23: 363–398, 2003.
6. Aggarwal BB, Sethi G, Baladandayuthapani V, Krishnan S, and Shishodia S. Targeting cell signaling pathways for drug discovery: An old lock needs a new key. *J Cell Biochem* 102: 580–592, 2007.
7. Aggarwal BB and Shishodia S. Molecular targets of dietary agents for prevention and therapy of cancer. *Biochem Pharmacol* 71: 1397–1421, 2006.
8. Aggarwal BB and Sung B. Pharmacological basis for the role of curcumin in chronic diseases: An age-old spice with modern targets. *Trends Pharmaco Sci* 30: 85–94, 2008.
9. Agullo G, Gamet–Payrastre L, Manenti S, Viala C, Remesy C, Chap H, and Payrastre B. Relationship between flavonoid structure and inhibition of phosphatidylinositol 3-kinase: A comparison with tyrosine kinase and protein kinase C inhibition. *Biochem Pharmacol* 53: 1649–1657, 1997.
10. Alessi DR, Andjelkovic M, Caudwell B, Cron P, Morrice N, Cohen P, and Hemmings BA. Mechanism of activation of protein kinase B by insulin and IGF-1. *EMBO J* 15: 6541–6551, 1996.
11. Alessi DR, Saito Y, Campbell DG, Cohen P, Sithanandam G, Rapp U, Ashworth A, Marshall CJ, and Cowley S. Identification of the sites in MAP kinase kinase-1 phosphorylated by p74raf-1. *EMBO J* 13: 1610–1619, 1994.
12. Altomare DA and Testa JR. Perturbations of the AKT signaling pathway in human cancer. *Oncogene* 24: 7455–7464, 2005.
13. Arora A and Scholar EM. Role of tyrosine kinase inhibitors in cancer therapy. *J Pharmacol Exp Ther* 315: 971–979, 2005.
14. Athar M, Back JH, Tang X, Kim KH, Kopelovich L, Bickers DR, and Kim AL. Resveratrol: A review of preclinical studies for human cancer prevention. *Toxicol Appl Pharmacol* 224: 274–283, 2007.
15. Atkinson C, Frankenfeld CL, and Lampe JW. Gut bacterial metabolism of the soy isoflavone daidzein: Exploring the relevance to human health. *Exp Biol Med (Maywood)* 230: 155–170, 2005.
16. Bader AG, Kang S, Zhao L, and Vogt PK. Oncogenic PI3K deregulates transcription and translation. *Nat Rev Cancer* 5: 921–929, 2005.
17. Barnett SF, Defeo–Jones D, Fu S, Hancock PJ, Haskell KM, Jones RE, Kahana JA, Kral AM, Leander K, Lee LL, Malinowski J, McAvoy EM, Nahas DD, Robinson RG, and Huber HE. Identification and characterization of pleckstrin-homology-domain-dependent and isoenzyme-specific Akt inhibitors. *Biochem J* 385: 399–408, 2005.
18. Blume–Jensen P and Hunter T. Oncogenic kinase signalling. *Nature* 411: 355–365, 2001.
19. Bogoyevitch MA and Fairlie DP. A new paradigm for protein kinase inhibition: Blocking phosphorylation without directly targeting ATP binding. *Drug Discovery Today* 12: 622–633, 2007.
20. Bonni A, Brunet A, West AE, Datta SR, Takasu MA, and Greenberg ME. Cell survival promoted by the Ras-MAPK signaling pathway by transcription-dependent and -independent mechanisms. *Science* 286: 1358–1362, 1999.
21. Bowman T, Garcia R, Turkson J, and Jove R. STATs in oncogenesis. *Oncogene* 19: 2474–2488, 2000.
22. Bradshaw L. Understanding piezoelectric quartz crystals. *RF time and freq* 8: 50–58, 2000.

23. Bromberg J and Darnell JE Jr. The role of STATs in transcriptional control and their impact on cellular function. *Oncogene* 19: 2468–2473, 2000.
24. Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, Hu LS, Anderson MJ, Arden KC, Blenis J, and Greenberg ME. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell* 96: 857–868, 1999.
25. Camandola S, Poli G, and Mattson MP. The lipid peroxidation product 4-hydroxy-2,3-nonenal increases AP-1-binding activity through caspase activation in neurons. *J Neurochem* 74: 159–168, 2000.
26. Cantley LC and Neel BG. New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase AKT pathway. *Proc Natl Acad Sci USA* 96: 4240–4245, 1999.
27. Cardone MH, Roy N, Stennicke HR, Salvesen GS, Franke TF, Stanbridge E, Frisch S, and Reed JC. Regulation of cell death protease caspase-9 by phosphorylation. *Science* 282: 1318–1321, 1998.
28. Castagne V and Clarke PG. Inhibitors of mitogen-activated protein kinases protect axotomized developing neurons. *Brain Res* 842: 215–219, 1999.
29. This reference has been deleted.
30. Chan AC, Irving BA, Fraser JD, and Weiss A. The zeta chain is associated with a tyrosine kinase and upon T-cell antigen receptor stimulation associates with ZAP-70, a 70-kDa tyrosine phosphoprotein. *Proc Natl Acad Sci USA* 88: 9166–9170, 1991.
31. Chan AC, Iwashima M, Turck CW, and Weiss A. ZAP-70: A 70kd protein-tyrosine kinase that associates with the TCR zeta chain. *Cell* 71: 649–662, 1992.
32. Chan K and Kan YW. Nrf2 is essential for protection against acute pulmonary injury in mice. *Proc Natl Acad Sci USA* 96: 12731–12736, 1999.
33. Chang F, Lee JT, Navolanic PM, Steelman LS, Shelton JG, Blalock WL, Franklin RA, and McCubrey JA. Involvement of PI3K/Akt pathway in cell cycle progression, apoptosis, and neoplastic transformation: A target for cancer chemotherapy. *Leukemia* 17: 590–603, 2003.
34. Chang F, Steelman LS, Lee JT, Shelton JG, Navolanic PM, Blalock WL, Franklin RA, and McCubrey JA. Signal transduction mediated by the Ras/Raf/MEK/ERK pathway from cytokine receptors to transcription factors: Potential targeting for therapeutic intervention. *Leukemia* 17: 1263–1293, 2003.
35. Cheetham GM. Novel protein kinases and molecular mechanisms of autoinhibition. *Curr Opin Struct Biol* 14: 700–705, 2004.
36. Chen C and Kong AN. Dietary cancer-chemopreventive compounds: From signaling and gene expression to pharmacological effects. *Trends Pharmacol Sci* 26: 318–326, 2005.
37. Chen Z, Gibson TB, Robinson F, Silvestro L, Pearson G, Xu B, Wright A, Vanderbilt C, and Cobb MH. MAP kinases. *Chem Rev* 101: 2449–2476, 2001.
38. Cho ES, Jang YJ, Kang NJ, Hwang MK, Kim YT, Lee KW, and Lee HJ. Cocoa procyanidins attenuate 4-hydroxynonenal-induced apoptosis of PC12 cells by directly inhibiting mitogen-activated protein kinase kinase 4 activity. *Free Radic Biol Med* 46: 1319–1327, 2009.
39. Choi YS, Ryu BK, Min HK, Lee SW, and Pak YK. Analysis of proteome bound to D-loop region of mitochondrial DNA by DNA-linked affinity chromatography and reverse-phase liquid chromatography/tandem mass spectrometry. *Ann NY Acad Sci* 1042: 88–100, 2005.
40. Clarke RB. p27KIP1 phosphorylation by PKB/Akt leads to poor breast cancer prognosis. *Breast Cancer Res* 5: 162–163, 2003.
41. Coffer PJ, Jin J, and Woodgett JR. Protein kinase B (c-Akt): A multifunctional mediator of phosphatidylinositol 3-kinase activation. *Biochem J* 335: 1–13, 1998.
42. Cohen P. Protein kinases—The major drug targets of the twenty-first century? *Nature Rev Drug Discov* 1: 309–315, 2002.
43. Cooper MA and Singleton VT. A survey of the 2001 to 2005 quartz crystal microbalance biosensor literature: Applications of acoustic physics to the analysis of biomolecular interactions. *J Mol Recog* 20: 154–184, 2007.
44. Crossthwaite AJ, Hasan S, and Williams RJ. Hydrogen peroxide-mediated phosphorylation of ERK1/2, Akt/PKB and JNK in cortical neurones: Dependence on $Ca^{(2+)}$ and PI3-kinase. *J Neurochem* 80: 24–35, 2002.
45. Crowder RJ and Freeman RS. Phosphatidylinositol 3-kinase and Akt protein kinase are necessary and sufficient for the survival of nerve growth factor-dependent sympathetic neurons. *J Neurosci* 18: 2933–2943, 1998.
46. Datta SR, Brunet A, and Greenberg ME. Cellular survival: A play in three Akts. *Genes Dev* 13: 2905–2927, 1999.
47. Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y, and Greenberg ME. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell* 91: 231–241, 1997.
48. David GS, Chino TH, and Reisfeld RA. Binding of proteins to CNBr-activated sepharose 4B. *FEBS Lett* 43: 264–266, 1974.
49. Davis RJ. Signal transduction by the JNK group of MAP kinases. *Cell* 103: 239–252, 2000.
50. Deeb O and Clare O. QSAR of aromatic substances: Protein tyrosine kinase inhibitory activity of flavonoid analogues. *Chem Biol Drug Des* 70: 437–449, 2007.
51. del Peso L, Gonzalez–Garcia M, Page C, Herrera R, and Nunez G. Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt. *Science* 278:687–689, 1997.
52. Denley A, Kang S, Karst U, and Vogt PK. Oncogenic signaling of class I PI3K isoforms. *Oncogene* 27: 2561–2574, 2008.
53. Dhillon AS, Meikle S, Yazici Z, Eulitz M, and Kolch W. Regulation of Raf-1 activation and signalling by dephosphorylation. *EMBO J* 21: 64–71, 2002.
54. Dixon MC. Quartz crystal microbalance with dissipation monitoring: Enabling real-time characterization of biological materials and their interactions. *J Biomole Techn* 9: 151–158, 2008.
55. Dixon SL, Smondyrev AM, Knoll EH, Rao SN, Shaw DE, and Friesner RA. PHASE: A new engine for pharmacophore perception, 3D QSAR model development, and 3D database screening: 1. Methodology and preliminary results. *J Comput Aided Mol Des* 20: 647–671, 2006.
56. Engh RA and Bossemeyer, D. Structural aspects of protein kinase control. Role of conformational flexibility. *Pharmacol Ther* 93: 99–111, 2002.
57. Ermakova SP, Choi BY, Choi HS, Kang BS, Bode AM, and Dong Z. The intermediate filament protein vimentin is a new target for epigallocatechin gallate. *J Biol Chem* 280: 16882–16890, 2005.
58. Ermakova SP, Kang BS, Choi BY, Choi HS, Schuster TF, Ma WY, Bode AM, and Dong Z. (-)-Epigallocatechin gallate

overcomes resistance to etoposide-induced cell death by targeting the molecular chaperone glucose-regulated protein 78. *Cancer Res* 66: 9260–9269, 2006.
59. Fabbro D, Ruetz S, Buchdunger E, Cowan–Jacob SW, Fendrich G, Liebetanz J, Mestan J, O'Reilly T, Traxler P, Chaudhuri B, Fretz H, Zimmermann J, Meyer T, Caravatti G, Furet P, and Manley PW: Protein kinases as targets for anticancer agents: From inhibitors to useful drugs. *Pharmaco Ther* 93: 79–98, 2002.
60. Fabian JR, Daar IO, and Morrison DK. Critical tyrosine residues regulate the enzymatic and biological activity of Raf-1 kinase. *Mol Cell Biol* 13: 7170–7179, 1993.
61. Fang JY and Richardson BC. The MAPK signalling pathways and colorectal cancer. *Lancet Oncol* 6: 322–327, 2005.
62. Fassihi A and Sabet R. QSAR study of p56lck protein tyrosine kinase inhibitory activity of flavonoid derivatives using MLR and GA-PLS. *Int J Mol Sci* 9: 1876–1892, 2008.
63. Favata MF, Horiuchi KY, Manos EJ, Daulerio AJ, Stradley DA, Feeser WS, Van Dyk DE, Pitts WJ, Earl RA, Hobbs F, Copeland RA, Magolda RL, Scherle PA, and Trzaskos JM. Identification of a novel inhibitor of mitogen-activated protein kinase kinase. *J Biol Chem* 273: 18623–18632, 1998.
64. Favreau LV and Pickett CB. The rat quinone reductase antioxidant response element: Identification of the nucleotide sequence required for basal and inducible activity and detection of antioxidant response element binding proteins in hepatoma and non-hepatoma cell lines. *J Biol Chem* 270: 24468–24474, 1995.
65. Ferriola PC, Cody V, and Middleton E Jr. Protein kinase C inhibition by plant flavonoids: Kinetic mechanisms and structure-activity relationships. *Biochem Pharmacol* 38: 1617–1624; 1989.
66. Fischmann TO, Smith CK, Mayhood TW, Myers JE, Reichert JP, Mannarino A, Carr D, Zhu H, Wong J, Yang RS, Le HV, and Madison VS. Crystal structures of MEK1 binary and ternary complexes with nucleotides and inhibitors. *Biochemistry* 48: 2661–2674, 2009.
67. Fry DW, Kraker AJ, McMichael A, Ambroso LA, Nelson JM, Leopold WR, Connors RW, and Bridges AJ. A specific inhibitor of the epidermal growth factor receptor tyrosine kinase. *Science* 265: 1093–1095, 1994.
68. Fujimura Y, Umeda D, Kiyohara Y, Sunada Y, Yamada K, and Tachibana H. The involvement of the 67 kDa laminin receptor-mediated modulation of cytoskeleton in the degranulation inhibition induced by epigallocatechin-3-O-gallate. *Biochem Biophys Res Commu* 348: 524–531, 2006.
69. Gamet–Payrastre L, Manenti S, Gratacap MP, Tulliez J, Chap H, and Payrastre B. Flavonoids and the inhibition of PKC and PI 3-kinase. *Gen Pharmacol* 32: 279–286, 1999.
70. Gershtein ES, Shatskaya VA, Ermilova VD, and Kushli NE. Phosphatidylinositol 3-kinase expression in human breast cancer. *Clin Chim Acta* 287: 59–67, 1999.
71. Gunn RM and Hailes HC. Insights into the PI3-K-PKB-mTOR signalling pathway from small molecules. *J Chem Biol* 1: 49–62, 2008.
72. Halliwell B, Zhao K, and Whiteman M. The gastrointestinal tract: A major site of antioxidant action? *Free Radic Res* 33: 819–830, 2000.
73. Hall–Jackson CA, Eyers PA, Cohen P, Goedert M, Boyle FT, Hewitt N, Plant H, and Hedge P. Paradoxical activation of Raf by a novel Raf inhibitor. *Chem Biol* 6: 559–568, 1999.
74. Han S, Zhou V, Pan S, Liu Y, Hornsby M, McMullan D, Klock HE, Haugen J, Lesley SA, Gray N, Caldwell J, and Gu XJ. Identification of coumarin derivatives as a novel class of allosteric MEK1 inhibitors. *Bioorg Med Chem Lett* 15: 5467–5473, 2005.
75. Hansch C, Hoekman D, and Gao H. Comparative QSAR: Toward a deeper understanding of chemicobiological interactions. *Chem Rev* 96: 1045–1075, 1996.
76. He Z, Tang F, Ermakova S, Li M, Zhao Q, Cho YY, Ma WY, Choi HS, Bode AM, Yang CS, and Dong Z. Fyn is a novel target of (-)-epigallocatechin gallate in the inhibition of JB6 Cl41 cell transformation. *Mol Carcinog* 47: 172–183, 2008.
77. Higdon JV and Frei B. Coffee and health: A review of recent human research. *Crit Rev Food Sci Nutr* 46: 101–123, 2006.
78. Hill MM and Hemmings BA. Inhibition of protein kinase B/Akt. Implications for cancer therapy. *Pharmacol Ther* 93: 243–251, 2002.
79. Hollman PC and Katan MB. Dietary flavonoids: Intake, health effects and bioavailability. *Food Chem Toxicol* 37: 937–942, 1999.
80. Horvath CM, Wen Z, and Darnell JE Jr. A STAT protein domain that determines DNA sequence recognition suggests a novel DNA-binding domain. *Genes Dev* 9: 984–994, 1995.
81. Hou DX. Potential mechanisms of cancer chemoprevention by anthocyanins. *Curr Mol Med* 3: 149–159, 2003.
82. Hou DX, Fujii M, Terahara N, and Yoshimoto N. Molecular mechanisms behind the chemopreventive effects of anthocyanidins (Review). *J Biomed Biotech* 5: 321–325, 2004.
83. Hou DX, Kai K, Li JJ, Lin S, Terahara N, Wakamatsu M, Fujii M, Young MR, and Colburn N. Anthocyanidins inhibit activator protein 1 activity and cell transformation: structure–activity relationship and molecular mechanisms. *Carcinogenesis* 25: 29–36, 2004.
84. Hou DX, Ose T, Lin S, Harazono K, Imamura I, Kubo M, Uto T, Terahara N, Yoshimoto M, and Fujii M. Anthocyanidins induce apoptosis in human promyelocytic leukemia cells: Structure–activity relationship and mechanisms involved. *Int J Oncol* 23: 705–712, 2003.
85. Hou DX, Yanagita T, Uto T, Masuzaki S, and Fujii M. Anthocyanidins inhibit cyclooxygenase-2 expression in LPS-evoked macrophages: Structure-activity relationship and molecular mechanisms involved. *Biochem Pharmacol* 70: 417–425, 2005.
86. Hsu TC, Young MR, Cmarik J, and Colburn NH. Activator protein 1 (AP-1)-and nuclear factor kappaB (NF-kappaB)-dependent transcriptional events in carcinogenesis. *Free Radic Biol Med* 28: 1338–1348, 2000.
87. Hu R and Kong AN. Activation of MAP kinases, apoptosis and nutrigenomics of gene expression elicited by dietary cancer-prevention compounds. *Nutrition* 20: 83–88, 2004.
88. Hwang MK, Kang NJ, Heo YS, Lee KW, and Lee HJ. Fyn kinase is a direct molecular target of delphinidin for the inhibition of cyclooxygenase-2 expression induced by tumor necrosis factor-alpha. *Biochem Pharmacol* 77: 1213–1222, 2009.
89. Hwang MK, Song NR, Kang NJ, Lee KW, and Lee HJ. Activation of phosphatidylinositol 3-kinase is required for tumor necrosis factor-alpha-induced upregulation of matrix metalloproteinase-9: Its direct inhibition by quercetin. *Int J Biochem Cell Biol* 41: 1592–1600, 2009.
90. Imada K and Leonard WJ. The Jak-STAT pathway. *Mol Immunol* 37: 1–11, 2000.
91. Inamdar NM, Ahn YI, and Alam J. The heme-responsive element of the mouse heme oxygenase-1 gene is an extended AP-1 binding site that resembles the recognition sequences for MAF and NF-E2 transcription factors. *Biochem Biophys Res Commun* 221: 570–576, 1996.

92. Jelić D, Mildner B, Kostrun S, Nujić K, Verbanac D, Culić O, Antolović R, and Brandt W. Homology modeling of human Fyn kinase structure: Discovery of rosmarinic acid as a new Fyn kinase inhibitor and *in silico* study of its possible binding modes. *J Med Chem* 50: 1090–1100, 2007.
93. Jin L, Pluske, S, Petrella EC, Cantin SM, Gorga JC, Rynkiewicz MJ, Pandey P, Strickler JE, Babine RE, Weaver DT, and Seidl KJ. The three-dimensional structure of the ZAP-70 kinase domain in complex with staurosporine: Implications for the design of selective inhibitors. *J Biol Chem* 279: 42818–42825, 2004.
94. Johnson LN. Protein kinase inhibitors: Contributions from structure to clinical compounds. *Q Rev Biophys* 42: 1–40, 2009.
95. Jung SK, Lee KW, Byun S, Kang NJ, Lim SH, Heo YS, Bode AM, Bowden GT, Lee HJ, and Dong Z. Myricetin suppresses UVB-induced skin cancer by targeting Fyn. *Cancer Res* 68: 6021–6029, 2008.
96. Kang NJ, Lee KW, Kwon JY, Hwang MK, Rogozin EA, Heo YS, Bode AM, Lee HJ, and Dong Z. Delphinidin attenuates neoplastic transformation in JB6 Cl41 mouse epidermal cells by blocking Raf/mitogen-activated protein kinase kinase/extracellular signal-regulated kinase signaling. *Cancer Prev Res (Phila Pa)* 1: 522–531, 2008.
97. Kang NJ, Lee KW, Lee DE, Rogozin EA, Bode AM, Lee HJ, and Dong Z. Cocoa procyanidins suppress transformation by inhibiting mitogen-activated protein kinase kinase. *J Biol Chem* 283: 20664–20673, 2008.
98. Kang NJ, Lee KW, Rogozin EA, Cho YY, Heo YS, Bode AM, Lee HJ, and Dong Z. Equol, a metabolite of the soybean isoflavone daidzein, inhibits neoplastic cell transformation by targeting the MEK/ERK/p90RSK/activator protein-1 pathway. *J Biol Chem* 282: 32856–32866, 2007.
99. Kang NJ, Lee KW, Shin BJ, Jung SK, Hwang MK, Bode AM, Heo YS, Lee HJ, and Dong Z. Caffeic acid, a phenolic phytochemical in coffee, directly inhibits Fyn kinase activity and UVB-induced COX-2 expression. *Carcinogenesis* 30: 321–330, 2009.
100. Karin M, Cao Y, Greten FR, and Li ZW. NF-kappaB in cancer: from innocent bystander to major culprit. *Nat Rev Cancer* 2: 301–310, 2002.
101. Karlsson R. SPR for molecular interaction analysis: A review of emerging application areas. *J Mol Recognit* 17: 151–161, 2004.
102. Khan N, Afaq F, Saleem M, Ahmad N, and Mukhtar H. Targeting multiple signaling pathways by green tea polyphenol (-)-epigallocatechin-3-gallate. *Cancer Res* 66: 2500–2505, 2006.
103. Kim JE, Kwon JY, Lee DE, Kang NJ, Heo YS, Lee KW, and Lee HJ. MKK4 is a novel target for the inhibition of tumor necrosis factor-alpha-induced vascular endothelial growth factor expression by myricetin. *Biochem Pharmacol* 77: 412–421, 2009.
104. Kim YC, Masutani H, Yamaguchi Y, Itoh K, Yamamoto M, and Yodoi J. Hemin-induced activation of the thioredoxin gene by Nrf2: A differential regulation of the antioxidant responsive element (ARE) by switch of its binding factors. *J Biol Chem* 276: 18399–18406, 2001.
105. Kinnings SL and Jackson RM. Binding site similarity analysis for the functional classification of the protein kinase family. *J Chem Inf Model* 49: 318–329, 2009.
106. Kirchmair J, Laggner C, Wolber G, and Langer T. Comparative analysis of protein-bound ligand conformations with respect to catalyst's conformational space subsampling algorithms. *J Chem Inf Model* 45: 422–430, 2005.
107. Klampfer L. Signal transducers and activators of transcription (STATs): Novel targets of chemopreventive and chemotherapeutic drugs. *Curr Cancer Drug Targets* 6: 107–121, 2006.
108. Klaus A and Birchmeier W. Wnt signalling and its impact on development and cancer. *Nat Rev Cancer* 8: 387–398, 2008.
109. Kobayashi S, Ji H, Yuza Y, Meyerson M, Wong KK, Tenen DG, and Halmos B. An alternative inhibitor overcomes resistance caused by a mutation of the epidermal growth factor receptor. *Cancer Res* 65: 7096–7101, 2005.
110. Kong AN, Yu R, Chen C, Mandlekar S, and Primiano T. Signal transduction events elicited by natural products: Role of MAPK and caspase pathways in homeostatic response and induction of apoptosis. *Arch Pharm Res* 23: 1–16, 2000.
111. Kubinyi H. Success stories of computer-aided design. In: *Computer Applications in Pharmaceutical Research and Development* (Ekins, S., ed.), Wiley-Interscience, New York, 2006, pp. 377–424.
112. Kumamoto T, Fujii M, and Hou DX. Myricetin directly targets JAK1 to inhibit cell transformation. *Cancer Lett* 275: 17–26, 2009.
113. Kumamoto T, Fujii M, and Hou DX. Akt is a direct target for myricetin to inhibit cell transformation. *Mol Cell Biochem* 332: 33–41, 2009.
114. Kumar CC and Madison V. AKT crystal structure and AKT-specific inhibitors. *Oncogene* 24: 7493–7501, 2005.
115. Kundu JK and Surh YJ. Cancer chemopreventive and therapeutic potential of resveratrol: Mechanistic perspectives. *Cancer Lett* 269: 243–261, 2008.
116. Kuo PC, Abe K, and Schroeder RA. Superoxide enhances interleukin 1beta-mediated transcription of the hepatocyte-inducible nitric oxide synthase gene. *Gastroenterology* 118: 608–618, 2000.
117. Lapenna S and Giordano A. Cell cycle kinases as therapeutic targets for cancer. *Nat Rev Drug Discov* 8: 547–556, 2009.
118. Laskowski RA, Gerick F, and Thornton JM. The structural basis of allosteric regulation in proteins. *FEBS Lett* 583: 1692–1698, 2009.
119. Lawrence DS and Niu J. Protein kinase inhibitors: The tyrosine-specific protein kinases. *Pharmacol Ther* 77: 81–114, 1998.
120. Lee KW, Kang NJ, Heo YS, Rogozin EA, Pugliese A, Hwang MK, Bowden GT, Bode AM, Lee HJ, and Dong Z. Raf and MEK protein kinases are direct molecular targets for the chemopreventive effect of quercetin, a major flavonol in red wine. *Cancer Res* 68: 946–955, 2008.
121. Lee KW, Kang NJ, Rogozin EA, Kim HG, Cho YY, Bode AM, Lee HJ, Surh YJ, Bowden GT, and Dong Z. Myricetin is a novel natural inhibitor of neoplastic cell transformation and MEK1. *Carcinogenesis* 28: 1918–1927, 2007.
122. Lee KW, Kang NJ, Rogozin EA, Oh SM, Heo YS, Pugliese A, Bode AM, Lee HJ, and Dong Z. The resveratrol analogue 3,5,3′,4′,5′-pentahydroxy-trans-stilbene inhibits cell transformation via MEK. *Int J Cancer* 123: 2487–2496, 2008.
123. Lin CH, Yeh SH, Lin CH, Lu KT, Leu TH, Chang WC, and Gean PW. A role for the PI-3 kinase signaling pathway in fear conditioning and synaptic plasticity in the amygdala. *Neuron* 31: 841–851, 2001.
124. Lindsley CW, Zhao Z, Leister WH, Robinson RG, Barnett SF, Defeo–Jones D, Jones RE, Hartman GD, Huff JR, Huber

HE, and Duggan ME. Allosteric Akt (PKB) inhibitors: Discovery and SAR of isozyme selective inhibitors. *Bioorg Med Chem Lett* 15: 761–764, 2005.
125. Liu Y and Gray NS. Rational design of inhibitors that bind to inactive kinase conformations. *Nature Chem Biol* 2: 358–364, 2006.
126. Los M, Maddika S, Erb B, and Schulze–Osthoff K. Switching Akt: From survival signaling to deadly response. *BioEssays* 31: 492–495, 2009.
127. Luo Z, Tzivion G, Belshaw PJ, Vavvas D, Marshall M, and Avruch J. Oligomerization activates c-Raf-1 through a Ras-dependent mechanism. *Nature* 383: 181–185, 1996.
128. Lyons JF, Wilhelm S, Hibner B, and Bollag G. Discovery of a novel Raf kinase inhibitor. *Endocr Relat Cancer* 8: 219–225, 2001.
129. Manning G, Whyte DB, Martinez R, Hunter T, and Sudarsanam S. The protein kinase complement of the human genome. *Science* 298: 1912–1934, 2002.
130. Maroney AC, Finn JP, Bozyczko–Coyne D, O'Kane TM, Neff NT, Tolkovsky AM, Park DS, Yan CY, Troy CM, and Greene LA. CEP-1347 (KT7515), an inhibitor of JNK activation, rescues sympathetic neurons and neuronally differentiated PC12 cells from death evoked by three distinct insults. *J Neurochem* 73: 1901–1912, 1999.
131. Martin GS. Cell signaling and cancer. *Cancer Cell* 4: 167–174, 2003.
132. Matter WF, Brown RF, and Vlahos CJ. The inhibition of phosphatidylinositol 3-kinase by quercetin and analogs. *Biochem Biophys Res Commun* 186: 624–631, 1992.
133. McCubrey JA, Steelman LS, Abrams SL, Lee JT, Chang F, Bertrand FE, Navolanic PM, Terrian DM, Franklin RA, D'Assoro AB, Salisbury JL, Mazzarino MC, Stivala F, and Libra M. Roles of the RAF/MEK/ERK and PI3K/PTEN/AKT pathways in malignant transformation and drug resistance. *Adv Enzyme Regul* 46: 249–279, 2006.
134. McCubrey JA, Steelman LS, Chappell WH, Abrams SL, Wong EW, Chang F, Lehmann B, Terrian DM, Milella M, Tafuri A, Stivala F, Libra M, Basecke J, Evangelisti C, Martelli AM, and Franklin RA. Roles of the Raf/MEK/ERK pathway in cell growth, malignant transformation and drug resistance. *Biochim Biophys Acta* 1773: 1263–1284, 2007.
135. McDonnell JM. Surface plasmon resonance: Towards an understanding of the mechanisms of biological molecular recognition. *Curr Opinion in Chem Biol* 5: 572–577, 2001.
136. McIntyre KW, Shuster DJ, Gillooly KM, Dambach DM, Pattoli MA, Lu P, Zhou XD, Qiu Y, Zusi FC, and Burke JR. A highly selective inhibitor of IκB kinase, BMS-345541, blocks both joint inflammation and destruction in collagen-induced arthritis in mice. *Arthritis Rheum* 48: 2652–2659, 2003.
137. McMahon G, Sun L, Liang C, and Tang C. Protein kinase inhibitors: Structural determinants for target specificity. *Curr Opin Drug Discov Dev* 1: 131–146, 1998.
138. Mencher SK and Wang LG. Promiscuous drugs compared to selective drugs (promiscuity can be a virtue). *BMC Clin Pharmacol* 5: 1–7, 2005.
139. Moinova HR and Mulcahy RT. An electrophile responsive element (EpRE) regulates beta-naphthoflavone induction of the human gammaglutamylcysteine synthetase regulatory subunit gene: Constitutive expression is mediated by an adjacent AP-1 site. *J Biol Chem* 273: 14683–14689, 1998.
140. Molecular Operating Environment (MOE). Chemical Computing Group. www.chemcomp.com., retrieved August 2007.
141. Mulcahy RT and Gipp JJ. Identification of a putative antioxidant response element in the 5′-flanking region of the human gammaglutamylcysteine synthetase heavy subunit gene. *Biochem Biophys Res Commun* 209: 227–233, 1995.
142. Murakami A, Ashida H, and Terao J. Multitargeted cancer prevention by quercetin. *Cancer Lett* 269: 315–325, 2008.
143. Myers MP, Pass I, Batty IH, Van der Kaay J, Stolarov JP, Hemmings BA, Wigler MH, Downes, CP, and Tonks NK. The lipid phosphatase activity of PTEN is critical for its tumour suppressor function. *Proc Natl Acad Sci USA* 95: 13513–13518, 1998.
144. Nagar B, Bornmann WG, Pellicena P, Schindler T, Veach DR, Miller WT, Clarkson B, and Kuriyan J. Crystal structures of the kinase domain of c-Abl in complex with the small molecule inhibitors PD173955 and imatinib (STI-571). *Cancer Res* 62: 4236–4243, 2002.
145. Newman DJ, Cragg GM, and Snader KM. Natural products as sources of new drugs over the period 1981–2002. *J Nat Prod* 66: 1022–1037, 2003.
146. Nicholson KM and Anderson NG. The protein kinase B/Akt signalling pathway in human malignancy. *Cell Signal* 14: 381–395, 2002.
147. Nkondjock A. Coffee consumption and the risk of cancer: An overview. *Cancer Lett* 18: 121–125, 2009.
148. Oblak M, Randic M, and Solmajer T. Quantitative structure–activity relationship of flavonoid analogues. 3. Inhibition of p56lck protein tyrosine kinase. *J Chem Inf Comput Sci* 40: 994–1001, 2000.
149. Ohren JF, Chen H, Pavlovsky A, Whitehead C, Zhang E, Kuffa P, Yan C, McConnell P, Spessard C, Banotai C, Mueller WT, Delaney A, Omer C, Sebolt–Leopold J, Dudley DT, Leung IK, Flamme C, Warmus J, Kaufman M, Barrett S, Tecle H, and Hasemann CA. Structures of human MAP kinase kinase 1 (MEK1) and MEK2 describe novel non-competitive kinase inhibition. *Nat Struct Mol Biol* 11: 1192–1197, 2004.
150. Olayioye MA, Neve RM, Lane HA, and Hynes NE. The ErbB signaling network: Receptor heterodimerization in development and cancer. *EMBO J* 19: 3159–3167, 2000.
151. Ong KC and Khoo HE. Biological effects of myricetin. *Gen Pharmacol* 29: 121–126, 1997.
152. Ozes ON, Mayo LD, Gustin JA, Pfeffer SR, Pfeffer LM, and Donner DB. NF-kappaB activation by tumour necrosis factor requires the Akt serine-threonine kinase. *Nature* 401: 82–85, 1999.
153. Pearson G, Bumeister R, Henry DO, Cobb MH, and White MA. Uncoupling Raf1 from MEK1/2 impairs only a subset of cellular responses to Raf activation. *J Biol Chem* 275: 37303–37306, 2000.
154. Perkinton M S, Sihra TS, and Williams RJ. $Ca(^{2+})$-permeable AMPA receptors induce phosphorylation of cAMP response element-binding protein through a phosphatidylinositol 3-kinase-dependent stimulation of the mitogen-activated protein kinase signaling cascade in neurons. *J Neurosci* 19: 5861–5874, 1999.
155. Pesu M, Laurence A, Kishore N, Zwillich SH, Chan G, and O'Shea JJ. Therapeutic targeting of Janus kinases. *Immunol Rev* 223: 132–142, 2008.
156. Petrelli A and Giordano S. From single- to multi-target drugs in cancer therapy: When a specificity becomes an advantage. *Curr Me. Chem* 15: 422–432, 2008.
156a. Pharmacophore Modeling and Analysis. Accelrys Inc. http://accelrys.com.

157. Phase. Schrodinger Inc. www.schrodinger.com., retrieved August 2007.
158. Phillips WA, StClair F, Munday AD, Thomas RJS, and Mitchell CA. Increased levels of phosphatidylinositol 3-kinase activity in colorectal tumors. *Cancer* 83: 41–47, 1998.
159. Phillips KS and Cheng Q. Recent advances in surface plasmon resonance based techniques for bioanalysis. *Anal Bioanal Chem* 387: 1831–1840, 2007.
160. Piccart–Gebhart MJ, Procter M, Leyland–Jones B, Goldhirsch A, Untch M, Smith I, Gianni L, Baselga J, Bell R, Jackisch C, Cameron D, Dowsett M, Barrios CH, Steger G, Huang CS, Andersson M, Inbar M, Lichinitser M, Lang I, Nitz U, Iwata H, Thomssen C, Lohrisch C, Suter TM, Ruschoff J, Suto T, Greatorex V, Ward C, Straehle C, McFadden E, Dolci MS, and Gelber RD. Trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer. *N Engl J Med* 353:1659–1672, 2005.
161. Ramos S. Cancer chemoprevention and chemotherapy: Dietary polyphenols and signalling pathways. *Mol Nutr Food Res* 52: 507–526, 2008.
162. Rane SG and Reddy EP. Janus kinases: Components of multiple signaling pathways. *Oncogene* 19: 5662–5679, 2000.
163. Rabindran SK, Discafani CM, Rosfjord EC, Baxter M, Floyd MB, Golas J, Hallett WA, Johnson BD, Nilakantan R, Overbeek E, Reich MF, Shen R, Shi X, Tsou HR, Wang YF, and Wissner A. Antitumor activity of HKI-272, an orally active, irreversible inhibitor of the HER-2 tyrosine kinase. *Cancer Res* 64: 3958–3965, 2004.
164. Ren Y and Smith A. Mechanism of metallothionein gene regulation by heme–hemopexin: Roles of protein kinase C, reactive oxygen species, and cis-acting elements. *J Biol Chem* 270: 23988–23995, 1995.
165. Ricci F, Volpe G, Micheli L, and Palleschi G. A review on novel developments and applications of immunosensors in food analysis. *Analyt Chimica Acta* 605: 111–129, 2007.
166. Rice–Evans C. Flavonoid antioxidants. *Curr Med Chem* 8: 797–807, 2001.
167. Rice–Evans C. Plant polyphenols: Free radical scavengers or chain-breaking antioxidants? *Biochem Soc Symp* 61: 103–116, 1995.
168. Rice–Evans CA, Miller NJ, and Paganga G. Structure–antioxidant activity relationships of flavonoids and phenolic acids. *Free Radic Biol Med* 20: 933–956, 1996.
169. Roberts PJ and Der CJ. Targeting the Raf-MEK-ERK mitogen-activated protein kinase cascade for the treatment of cancer. *Oncogene* 26: 3291–3310, 2007.
170. Romashkova JA and Makarov SS. NF-kappaB is a target of AKT in anti-apoptotic PDGF signalling. *Nature* 401: 86–90, 1999.
171. Rommel C, Clarke BA, Zimmermann S, Nuñez L, Rossman R, Reid K, Moelling K, Yancopoulos GD, and Glass DJ. Differentiation stage-specific inhibition of the Raf-MEK-ERK pathway by Akt. *Science* 286: 1738–1741, 1999.
172. Rushmore TH, King RG, Paulson KE, and Pickett CB. Regulation of glutathione S-transferase Ya subunit gene expression: Identification of a unique xenobiotic-responsive element controlling inducible expression by planar aromatic compounds. *Proc Natl Acad Sci USA* 87: 3826–3830, 1990.
173. Salmond RJ, Filby A, Qureshi I, Caserta S, and Zamoyska R. T-cell receptor proximal signaling via the Src-family kinases, Lck and Fyn, influences T-cell activation, differentiation, and tolerance. *Immunol Rev* 228: 9–22, 2009.
174. Salvesen GS and Riedl SJ. Caspase inhibition, specifically. *Structure* 15: 513–514, 2007.
175. Sasaki H, Sato H, Kuriyama–Matsumura K, Sato K, Maebara K, Wang H, Tamba M, Itoh K, Yamamoto M, and Bannai S. Electrophile response element-mediated induction of the cystine/glutamate exchange transporter gene expression. *J Biol Chem* 277: 44765–44771, 2002.
176. Schlessinger J. Cell signaling by receptor tyrosine kinases. *Cell* 103: 211–225, 2000.
177. Schroeter H, Boyd C, Spencer JPE, Williams RJ, Cadenas E, and Rice–Evans C. MAPK signaling in neurodegeneration: Influences of flavonoids and of nitric oxide. *Neurobiol Aging* 23: 861–880, 2002.
178. Schroeter H, Spencer JP, Rice–Evans C, and Williams RJ. Flavonoids protect neurons from oxidized low-density-lipoprotein-induced apoptosis involving c-Jun N-terminal kinase (JNK), c-Jun and caspase-3. *Biochem J* 358: 547–557, 2001.
179. Schweizer A. Roschitzki–Voser H, Amstutz P, Briand C, Gulotti–Georgieva M, Prenosil E, Binz HK, Capitani G, Baici A, Plückthun A, and Grütter MG. Inhibition of caspase-2 by a designed ankyrin repeat protein: Specificity, structure, and inhibition mechanism. *Structure* 15: 625–636, 2007.
180. Sebolt–Leopold JS. Development of anticancer drugs targeting the MAP kinase pathway. *Oncogene* 19: 6594–6599, 2000.
181. Sebolt–Leopold JS and Herrera R. Targeting the mitogen-activated protein kinase cascade to treat cancer. *Nat Rev Cancer* 4: 937–947, 2004.
182. Seger R and Krebs EG. The MAPK signaling cascade. *FASEB J* 9: 726–735, 1995.
183. Shakibaei M, Harikumar KB, and Aggarwal BB. Resveratrol addiction: To die or not to die. *Mol Nutr Food Res* 53: 115–128, 2009.
184. Shchemelinin I, Šefc L, and Nečas E. Protein kinases, their functions and implication in cancer and other diseases. *Folia Biol (Praha)* 52: 81–101, 2006.
185. She QB, Ma WY, Wang M, Kaji A, Ho CT, and Dong Z. Inhibition of cell transformation by resveratrol and its derivatives: Differential effects and mechanisms involved. *Oncogene* 22: 2143–2150, 2003.
186. Shelton JG, Moye PW, Steelman LS, Blalock WL, Lee JT, Franklin RA, McMahon M, and McCubrey JA. Differential effects of kinase cascade inhibitors on neoplastic and cytokine-mediated cell proliferation. *Leukemia* 17: 1765–1782, 2003.
187. Shiba Y, Kinoshita T, Chuman H, Taketani Y, Takeda E, Kato Y,| Naito M, Kawabata K, Ishisaka A, Terao J, and Kawai Y. Flavonoids as substrates and inhibitors of myeloperoxidase: Molecular actions of aglycone and metabolites. *Chem Res Toxico* 21: 1600–1609, 2008.
188. Shim JH, Choi HS, Pugliese A, Lee SY, Chae JI, Choi BY, Bode AM, and Dong Z. (-)-Epigallocatechin gallate regulates CD3-mediated T cell receptor signaling in leukemia through the inhibition of ZAP-70 kinase. *J Biol Chem* 283: 28370–28379, 2008.
189. Shishodia S and Aggarwal BB. Guggulsterone inhibits NF-kappaB and IkappaBalpha kinase activation, suppresses expression of anti-apoptotic gene products, and enhances apoptosis. *J Biol Chem* 279: 47148–47158, 2004.
190. Shuai K, Horvath CM, Huang LH, Qureshi SA, Cowburn D, and Darnell JE Jr. Interferon activation of the transcription factor Stat91 involves dimerization through SH2-phosphotyrosyl peptide interactions. *Cell* 76: 821–828, 1994.

191. Smyth LA and Collins I. Measuring and interpreting the selectivity of protein kinase inhibitors. *J Chem Biol* 2: 131–151, 2009.
192. Soh Y, Jeong KS, Lee IJ, Bae MA, Kim YC, and Song BJ. Selective activation of the c-Jun N-terminal protein kinase pathway during 4-hydroxynonenal-induced apoptosis of PC12 cells. *Mol Pharmacol* 58: 535–541, 2000.
193. Spencer JP, Rice–Evans C, and Williams RJ. Modulation of pro-survival Akt/PKB and ERK1/2 signalling cascades by quercetin and its *in vivo* metabolites underlie their action on neuronal viability. *J Biol Chem* 278: 34783–34793, 2003.
194. Spencer JP, Schroeter H, Crossthwaithe AJ, Kuhnle G., Williams RJ, and Rice-Evans C. Contrasting influences of glucuronidation and *O*-methylation of epicatechin on hydrogen peroxide-induced cell death in neurons and fibroblasts. *Free Radic Biol Med* 31: 1139–1146, 2001.
195. Spencer JP, Schroeter H, Kuhnle G, Srai SK, Tyrrell RM, Hahn U, and Rice–Evans C. Epicatechin and its *in vivo* metabolite, 3V-O-methyl epicatechin, protect human fibroblasts from oxidative-stress-induced cell death involving caspase-3 activation. *Biochem J* 354: 493–500, 2001.
196. Stiewe T. The p53 family in differentiation and tumorigenesis. *Nat Rev Cancer* 7: 165–168, 2007.
197. Surh YJ. Cancer chemoprevention with dietary phytochemicals. *Nat Rev Cancer* 3: 768–780. 2003;
198. Sweatt JD. Memory mechanisms: The yin and yang of protein phosphorylation. *Curr Biol* 11: R391–R394, 2001.
199. Tachibana H, Koga K, Fujimura Y, and Yamada K. A receptor for green tea polyphenol EGCG. *Nat Struct Mol Biol* 11: 380–381, 2004.
200. Teillet F, Boumendje A, Boutonnat J, and Ronot X. Flavonoids as RTK inhibitors and potential anticancer agents. *Med Res Reviews* 28: 715–745, 2008.
201. Torres M and Forman HJ. Redox signaling and the MAP kinase pathways. *Biofactors* 17: 287–296, 2003.
202. Traxler P and Furet P. Strategies toward the design of novel and selective protein tyrosine kinase inhibitors. *Pharmacol Ther* 82: 195–206, 1999.
203. Tsuji Y, Ayaki H, Whitman SP, Morrow CS, Torti SV, and Torti FM. Coordinate transcriptional and translational regulation of ferritin in response to oxidative stress. *Mol Cell Biol* 20: 5818–5827, 2000.
204. Turkson J and Jove R. STAT proteins: Novel molecular targets for cancer drug discovery. *Oncogene* 19: 6613–6626, 2000.
205. Ueda S, Masutani H, Nakamura H, Tanaka T, Ueno M, and Yodoi J. Redox control of cell death. *Antioxid Redox Signal* 4: 405– 414, 2002.
206. VanScyoc WS, Holdgate GA, Sullivan JE, and Ward WH. Enzyme kinetics and binding studies on inhibitors of MEK protein kinase. *Biochemistry* 47: 5017–5027, 2008.
207. Venter JC, Adams MD, Myers EW, *et al.* The sequence of the human genome. *Science* 291: 1304–1351, 2001.
208. Vlahos CJ, Matter WF, Hui KY, and Brown RF. A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). *J Biol Chem* 18: 5241–5248, 1994.
209. Virgili F and Marino M. Regulation of cellular signals from nutritional molecules: A specific role for phytochemicals, beyond antioxidant activity. *Free Radical Biol Med* 45: 1205–1216, 2008.
210. Wagner EF and Nebreda AR. Signal integration by JNK and p38 MAPK pathways in cancer development. *Nat Rev Cancer* 9: 537–549, 2009.
211. Walker EH, Pacold ME, Perisic O, Stephens L, Hawkins PT, Wymann MP, and Williams RL. Structural determinants of phosphoinositide 3-kinase inhibition by wortmannin, LY294002, quercetin, myricetin, and staurosporine. *Mol Cell* 6: 909–919, 2000.
212. Wan PT, Garnett MJ, Roe SM, Lee S, Niculescu–Duvaz D, Good VM, Jones CM, Marshall CJ, Springer CJ, Barford D, and Marais R. Mechanism of activation of the RAF-ERK signaling pathway by oncogenic mutations of B-RAF. *Cell* 116: 855–867, 2004.
213. Wang LS and Stoner GD. Anthocyanins and their role in cancer prevention. *Cancer Lett* 269: 281–290, 2008.
214. Wegener J. Janshoff A, and Steinem C. The quartz crystal microbalance as a novel means to study cell–substrate interactions *in situ. Cell Biochem Biophy* 34: 121–131, 2001.
215. Weisburger JH. Chemopreventive effects of cocoa polyphenols on chronic diseases. *Exp Biol Med (Maywood)* 226: 891–897, 2001.
216. Wen Z and Darnell JE Jr. Mapping of Stat3 serine phosphorylation to a single residue (727) and evidence that serine phosphorylation has no influence on DNA binding of Stat1 and Stat3. *Nucleic Acids Res* 25: 2062–2067, 1997.
217. Wen Z, Zhong Z, and Darnell JE Jr. Maximal activation of transcription by Stat1 and Stat3 requires both tyrosine and serine phosphorylation. *Cell* 82: 241–250, 1995.
218. Whitmarsh AJ and Davis RJ. Role of mitogen-activated protein kinase kinase 4 in cancer. *Oncogene* 26: 3172–3184, 2007.
219. Williams RJ, Spencer JPE, and Rice–Evans C. Flavonoids: Antioxidants or signaling molecules? *Free Radical Biol Med* 36: 838–849, 2004.
220. Wolber G and Kosara R. Pharmacophores from macromolecular complexes with Ligand Scout. In: *Pharmacophores and Pharmacophore Searches*, (vol. 32) (Langer, T. and Hoffmann, R.D., eds), Wiley-VCH, 2006, pp. 131–150.
221. Wolber G and Langer T. Ligand Scout: 3D pharmacophores derived from protein-bound ligands and their use as virtual screening filters. *J Chem Inf Model* 45: 160–169, 2005.
222. Wolber G, Seidel T, Bendix F, and Langer T. Molecule-pharmacophore superpositioning and pattern matching. *Drug Discov Today* 13: 23–29, 2008.
223. Wu X, Senechal K, Neshat MS, Whang YE, and Sawyers CL. The PTEN/MMAC1 tumor suppressor phosphatase functions as a negative regulator of the phosphoinositide 3-kinase/Akt pathway. *Proc Natl Acad Sci USA* 95: 15587–15591, 1998.
224. Xu X, Sun YL, and Hoey T. Cooperative DNA binding and sequence-selective recognition conferred by the STAT amino-terminal domain. *Science* 273: 794–797, 1996.
225. Yamaoka K, Saharinen P, Pesu M, Holt VE 3rd, Silvennoinen O, and O'Shea JJ. The Janus kinases (Jaks). *Genome Biol* 5: 253.1–253.6, 2004.
226. Yan J, Roy S, Apolloni A, Lane A, and Hancock JF. Ras isoforms vary in their ability to activate Raf-1 and phosphoinositide 3-kinase. *J Biol Chem* 273: 24052–24056, 1998.
227. Yang, CS, Maliakal P, and Meng X. Inhibition of carcinogenesis by tea. *Annu Rev Pharmacol Toxicol* 42: 25–54. 2002.
228. Yang, CS, and Wang ZY. Tea and Cancer. *J Natl Cancer Inst* 85: 1038–1049, 1993.
229. Yeung K, Seitz T, Li S, Janosch P, McFerran B, Kaiser C, Fee F, Katsanakis KD, Rose DW, Mischak H, Sedivy

JM, and Kolch W. Suppression of Raf-1 kinase activity and MAP kinase signalling by RKIP. *Nature* 401: 173–177, 1999.
230. Yoshioka K, Deng T, Cavigelli M, and Karin M. Antitumor promotion by phenolic antioxidants: Inhibition of AP-1 activity through induction of Fra expression. *Proc Natl Acad Sci USA* 92: 4972–4976, 1995.
231. Yuan JP, Wang JH, and Liu X. Metabolism of dietary soy isoflavones to equol by human intestinal microflora. Implications for health. *Mol Nutr Food Res* 51: 765–781, 2007.
232. Yuan TL and Cantley LC. PI3K pathway alterations in cancer: Variations on a theme. *Oncogene* 27: 5497–5510, 2008.
233. Zaveri NT. Green tea and its polyphenolic catechins: Medicinal uses in cancer and noncancer applications. *Life Sci* 78: 2073–2080, 2006.
234. Zha J, Harada H, Yang E, Jockel J, and Korsmeyer SJ. Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X(L). *Cell* 87: 619–628, 1996.
235. Zhang J, Yang PL, Nathanael S, and Gray NS: Targeting cancer with small molecule kinase inhibitors. *Nat Rev Cancer* 9: 28–39, 2009.

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Abbreviations Used

Aβ = amyloid beta
Akt/PKB = Akt/protein kinase B
AML = chronic lymphocytic leukemia
AP-1 = activator protein-1
ATF-2 = activating transcription factor-2
ATP = adenosine triphosphate
Bad = Bcl-2-associated death promoter
Bcl-2 = B-cell lymphoma 2
Bcl-x_L = B-cell lymphoma-extra large
BIA = biomolecular interaction analysis
CD3 = cluster of differentiation 3
CLL = acute myelocytic leukemia
CNBr = cyanogen bromide
COX-2 = cyclooxygenase-2
CREB = cyclic AMP (cAMP)-response element binding protein
DMSO = dimethyl sulfoxide
EGCG = (-)-epigallocatechin 3-gallate
EGF = epidermal growth factor
EGFR = EGF receptor
ERK = extracellular signal-regulated kinase
FLT3 = FMS-like tyrosine kinase 3
GST = glutathione-S-transferase
HER2 = human epidermal growth factor receptor 2
HO-1 = heme oxygenase-1
IL-2 = interleukin-2
iNOS = inducible nitric oxide synthase
JAK = janus kinase
JH = JAK homology
JNK = c-Jun N-terminal kinase
Kd = dissociation constant
LGL = large granular lymphocyte
MAPK = mitogen-activated protein kinase
MEK = mitogen-activated protein kinase kinase
MKK4 = MAPK kinase 4
MMP-9 = matrix metalloproteinase-9
MOE = Molecular Operating Environment
MSK = mitogen- and stress-activated protein kinase
NF-κB = nuclear factor-kappa B
NQO1 = NADPH: quinone oxidoreductase
p90RSK = p90 ribosomal S6 kinase
PDGF = platelet-derived growth factor
PDK = pyruvate dehydrogenase kinase
PH = pleckstrin homology
PI3K = phosphatidylinositol 3-kinase
PKA = protein kinase A
PKC = protein kinase C
QCM = quartz crystal microbalance
QSAR = quantitative structure-activity relationship
RONS = reactive oxygen-nitrogen species
ROS = reactive oxygen species
RSVL = resveratrol
SDS-PAGE = sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SH = Src homology
SPR = surface plasmon resonance
Src = sarcoma
STAT = signal transducers and activators of transcription
TNF-α = tumor necrosis factor-alpha
TPA = 12-*O*-tetradecanoylphorbol-13-acetate
TSM = thickness shear mode
Tyk2 = tyrosine kinase 2
UVB = ultra violet B
VEGF = vascular endothelial growth factor
ZAP-70 = ζ chain-associated 70-kDa protein.

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2. Darkhan Utepbergenov, Urszula Derewenda, Natalya Olekhnovich, Gabriela Szukalska, Budhaditya Banerjee, Michael K. Hilinski, Deborah A. Lannigan, P. Todd Stukenberg, Zygmunt S. Derewenda. 2012. Insights into the Inhibition of the p90 Ribosomal S6 Kinase (RSK) by the Flavonol Glycoside SL0101 from the 1.5 Å Crystal Structure of the N-Terminal Domain of RSK2 with Bound Inhibitor. *Biochemistry* **51**:33, 6499-6510. [CrossRef]
3. Graziano Lolli, Giorgio Cozza, Marco Mazzorana, Elena Tibaldi, Luca Cesaro, Arianna Donella-Deana, Flavio Meggio, Andrea Venerando, Cinzia Franchin, Stefania Sarno, Roberto Battistutta, Lorenzo A. Pinna. 2012. Inhibition of Protein Kinase CK2 by Flavonoids and Tyrphostins. A Structural Insight. *Biochemistry* **51**:31, 6097-6107. [CrossRef]
4. Lunawati L. Bennett, Stephen Rojas, Teresa Seefeldt. 2012. Role of Antioxidants in the Prevention of Cancer. *Journal of Experimental & Clinical Medicine* **4**:4, 215-222. [CrossRef]
5. Feng Jin, Dan Gao, Cunlong Zhang, Feng Liu, Bizhu Chu, Yan Chen, Yu Zong Chen, Chunyan Tan, Yuyang Jiang. 2012. Exploration of 1-(3-chloro-4-(4-oxo-4H-chromen-2-yl)phenyl)-3-phenylurea Derivatives as Selective Dual Inhibitors of Raf1 and JNK1 Kinases for Anti-tumor Treatment. *Bioorganic & Medicinal Chemistry* . [CrossRef]
6. Salvatore Chirumbolo. 2012. Plant phytochemicals as new potential drugs for immune disorders and cancer therapy: really a promising path?. *Journal of the Science of Food and Agriculture* n/a-n/a. [CrossRef]
7. Akira Murakami, Kohta Ohnishi. 2012. Target molecules of food phytochemicals: Food science bound for the next dimension. *Food & Function* . [CrossRef]
8. Wayne BidlackNutritional Genomics 77-138. [CrossRef]
9. C Spagnuolo, C Cerella, M Russo, S Chateauvieux, M Diederich, G L Russo. 2011. Quercetin downregulates Mcl-1 by acting on mRNA stability and protein degradation. *British Journal of Cancer* **105**:2, 221-230. [CrossRef]