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## Investigation of the Role of Dietary Flavonoids on Cell Death: Evidence to Support a Non-Classical Apoptotic Mechanism

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INVESTIGATION OF THE ROLE OF DIETARY FLAVONOIDS ON CELL DEATH:  
EVIDENCE TO SUPPORT A NON-CLASSICAL APOPTOTIC MECHANISM

A Thesis

Presented to

The Faculty of the Department of Biological Sciences

San José State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Tetiana Lialiutska

December 2012

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The Designated Thesis Committee Approves the Thesis Titled

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## ABSTRACT

### INVESTIGATION OF THE ROLE OF DIETARY FLAVONOIDS ON CELL DEATH: EVIDENCE TO SUPPORT A NON-CLASSICAL APOPTOTIC MECHANISM

by Tetiana Lialiutska

Flavonoids are polyphenolic compounds widely distributed in plants and have been shown to be cytotoxic to cancer cells. Our lab has previously shown that some flavonoids are able to induce cytotoxicity and inhibit caspase activity in human breast cancer cell lines. The goal of our study was to identify the specific molecular pathways required for flavonoid-induced cytotoxicity. MDA-MB-231 cells, mouse embryonic fibroblasts (MEFs) lacking caspase 3 and 7, and primary baby mouse kidney epithelial cells lacking Bax and Bak were cultured. Flavonoid-treated MDA-MB-231 cells showed cytochrome c release. We detected caspase 3 activation and PARP cleavage in MDA-MB-231 cells after treatment with some flavonoids. Quercetin did not activate caspase 3 but induced PARP cleavage at early time points. These results suggested that cytotoxicity in breast cancer cells involved caspase-independent mechanisms. To further test this, we treated wild-type and caspase-deficient MEFs with various flavonoids and performed cell death assays. Flavonoid-induced cell death in caspase-deficient MEFs and Bax/Bak deficient cell lines was similar to wild-type cells. Taken together, our results suggested that flavonoids induce cell death through caspase-independent mechanisms.

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## **List of Abbreviations**

ATCC: american type culture collection

AIF: apoptosis inducing factor

AP-1: activation protein – 1

Bcl-2: B cell lymphoma

BMKs: baby mouse kidney cells

CARD: caspase recruitment domain

CI-PCD: caspase-independent programmed cell death

DED: death effector domain

DISC: death-inducing signaling complex

DMEM: Dulbecco's modification of eagle's medium

DMSO: dimethyl sulfoxide

EC: epicatechin

EGC: epigallocatechin

EGCG: epigallocatechin-3-gallate

EndoG: endonuclease G

ER: estrogen receptor

FBS: fetal bovine serum

GAPDH: glyceraldehyde 3-phosphate dehydrogenase

HRP: horseradish peroxidase

ICAD: inhibitor of caspase-activated DNase

M1: marker 1

MEFs: mouse embryonic fibroblasts

MOMP: mitochondria outer membrane permeabilization

mTOR: mammalian target of rapamycin

NF- $\kappa$ B: nuclear factor kappa B

NLR: NOD-like receptor

PARP: poly-ADP-ribose polymerase

PBS: phosphate buffered saline

PI3K: phosphatidylinositol 3'-kinase

PtdIns(4,5)P<sub>2</sub>: phosphatidylinositol 4,5-bisphosphate

PtdIns(3,4,5)P<sub>3</sub>: phosphatidylinositol (3,4,5)-triphosphate

RIPK1: receptor-interacting protein 1 kinase

RLR: RIG-I-like receptor

ROS: reactive oxygen species

SDS-PAGE: Sodium Dodecylsulfate Polyacrylamide Gel Electrophoresis

STR: staurosporine

tBid: truncated Bid

TNF: tumor necrosis factor

TLR: toll-like receptor

XIAP: X-linked inhibitor of apoptosis protein

## **Introduction**

Cancer is one of the most serious health problems in the United States and many other countries in the world. The American Cancer Society estimates that by the end of 2012, there will be 1,638,910 new cancer cases and 577,190 deaths due to cancer in the United States (Siegel, Naishadham, & Jemal, 2012). Breast cancer is the second most common cause of cancer-related deaths in women (Chan & Morris, 2006; DeSantis, Siegel, Bandi, & Jemal, 2011). Many chemopreventive and chemotherapeutic drugs have noticeable side effects; therefore, the necessity to find safer therapeutic and preventive agents is still an important topic of research (Yao, 2011).

Flavonoids are bioactive compounds that can be found in apples, berries, tea chocolate, grapes, red wine, and other fruit and vegetables (Beecher, 2003). These compounds have many important biological properties. Various flavonoids possess anti-inflammatory, anti-tumor, and anti-oxidant activities (Atmani, 2009; Kanadaswami et al., 2005; Le Marchand, 2002; Ramos, 2007). Flavonoids are able to induce cell death in cultured cancer cells but not normal cells (Das, Banik, & Ray, 2010; Ramos, 2007).

Cell death in multicellular organisms occurs by different mechanisms, including apoptosis, autophagy, necrosis, and necroptosis (Duprez, Wirawan, Vanden Berghe, & Vandenabeele, 2009). Autophagy is a catabolic pathway that allows cells to degrade their own components. It is characterized by formation of autophagosomes, degradation of cytoplasmic contents, and slight chromatin condensation. Necrosis is usually described as uncontrolled cell death. It is characterized by cellular edema, disruption of the plasma membrane, release of cellular components, and induction of an inflammatory



response (Fink & Cookson, 2005). Accumulating evidence now supports the existence of programmed necrosis or necroptosis. The kinase activity of receptor-interacting protein 1 (RIPK1) and 3 (RIPK3) is required for death receptor-mediated necroptosis (Galluzzi & Kroemer, 2008).

Apoptosis is a form of programmed cell death associated with specific morphological features, such as chromatin condensation, nuclear fragmentation, and plasma membrane blebbing. Caspases (cysteine proteases) are involved in extrinsic (death receptor-mediated) or intrinsic (mitochondria-mediated) apoptotic cascade (Kim, Emi, & Tanabe, 2005). Apoptosis can also be initiated without caspase activation (Constantinou, 2009).

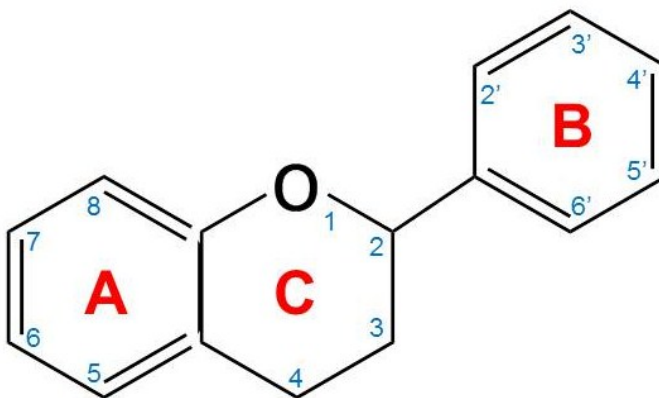
Numerous groups have reported that flavonoids induce cytotoxicity in cancer cells through classical apoptosis (Chien, 2009; Choi, Ahn, & Bae, 2009; Chung, 2010; Jin, Zhang, Kang, Wang, & Zhao, 2010; Kang et al., 2009; Shim et al., 2007), but data from our lab suggest that flavonoids inhibit caspase 1, 3, and 7 activity and that inhibition can be caspase-specific (White et al., 2012). The mechanism of flavonoid-induced cell death in breast cancer is still unclear.

In this study, we sought to determine the apoptotic mechanism involved in flavonoid-induced cell death. Understanding the signaling pathways that control flavonoid-induced cytotoxicity in tumors is critical to improve anti-cancer therapy.

## Literature Review

### Flavonoids: Structure, Classification, Anti-Tumor Activities

Flavonoids are a group of polyphenols that are widely distributed in nature. Over 800 different structures have been identified and classified into 10 chemical groups (Atmani, 2009; Ramos, 2007). Flavonoids consist of two aromatic rings connected by a three-carbon “bridge” that may be part of six-member heterocyclic pyran ring (Beecher, 2003) (Figure 1). Flavonoids are further divided into six subclasses, based on the connection of the B (aromatic) ring to the C (heterocyclic) ring.



*Figure 1.* General structure of flavonoids (Modified from Atmani, 2009).

Within each subclass, individual compounds are characterized by specific hydroxylation and conjugation patterns (Beecher, 2003; Yao, 2011). Flavonoids are prevalent in the human diet and are found in fruits, vegetables, soy products, tea, and red wine (Le Marchand, 2002) (Figure 2).

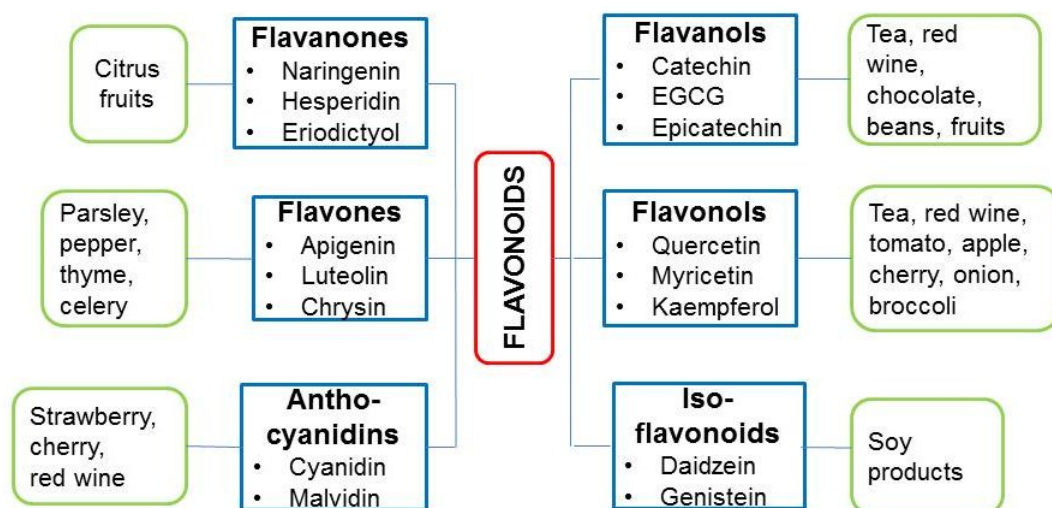


Figure 2. Members of flavonoids and their food sources.

Many investigations *in vitro* and *in vivo* have shown that members of the flavonoid family have anti-inflammatory, anti-tumor, and anti-oxidant activities (Atmani, 2009; Kale, 2008; Le Marchand, 2002). Cell culture and animal models have shown that flavonoids possess a variety of anticancer activities, such as inhibition of cell growth and kinase activity, induction of apoptosis, and suppression of secretion of matrix metalloproteinases (Kanadaswami et al., 2005; Ramos, 2007).

Quercetin, mostly present in apples and onions, is one of the most studied flavonoids and is a major component of human diet (Jan et al., 2010). Quercetin has shown pro-apoptotic properties in most commonly used cell lines through increasing intracellular reactive oxygen species (ROS) levels (Gibellini et al., 2011), cell cycle arrest (Chien, 2009; Chou, 2010), and activation of caspases (Chien, 2009; Galluzzo et al., 2009; Jung et al., 2010; Williams, Spencer, & Rice-Evans, 2004). Luteolin is a flavone present in celery, parsley, green pepper, artichoke, and other herbs and vegetables (Way,

Kao, & Lin, 2005). Luteolin is one of the most effective inhibitors of tumor cell proliferation among other flavonoids (Seelinger, Merfort, Wolfle, & Schempp, 2008). Luteolin's anti-cancer properties include induction of apoptosis, inhibition of cell proliferation, and inhibition of metastasis and angiogenesis. Luteolin can also block cancer development by suppressing cell survival pathways, such as phosphatidylinositol 3'-kinase (PI3K)/Akt, nuclear factor kappa B (NF- $\kappa$ B), and X-linked inhibitor of apoptosis protein (XIAP), and by stimulating extrinsic and intrinsic apoptotic pathways (Lin, Shi, Wang, & Shen, 2008; Seelinger et al., 2008; Shi, Ong, & Shen, 2005).

Apigenin is present in fruits, such as oranges and grapefruits, chamomile tea, parsley, and onions. It has been reported to be effective against breast, skin, prostate, colon, and certain hematological malignancies. Apigenin induces growth inhibition, cell cycle arrest, and apoptosis (Shukla & Gupta, 2010; Zheng, Chiang, & Lin, 2005).

Chrysin belongs to flavones and is widely present in herbs and vegetables, such as pepper, thyme, celery, and parsley (Ramos, 2007). Chrysin inhibits proliferation and induces apoptosis in most cancer cells. It acts via caspase activation and inactivation of Akt signaling (Khoo, Chua, & Balaram, 2010; Li, Huang, Ong, Yang, & Shen, 2010; Li et al., 2011; Woo, Jeong, Park, & Kwon, 2004).

Green tea is known to be rich in flavonoids called catechins, specifically epigallocatechin-3-gallate (EGCG), epigallocatechin (EGC), epicatechin-3-gallate (ECG), and epicatechin (EC). Anti-tumor activities of EGCG result from inhibition of protein kinase activity, blockage of activation protein – 1 (AP-1) and NF- $\kappa$ B activation, inhibition of cell proliferation, induction of apoptosis (Dou, 2009; Kale, 2008; Lee et al.,

2010). Genistein and daidzein belong to the isoflavone family and are often found in soy products. The isoflavonoids are considered to be phytoestrogens because of their structural similarity to the mammalian steroid hormone 17 $\beta$ -estradiol. They are able to bind and activate the estrogen receptor (ER) (Sakamoto, Horiguchi, Oguma, & Kayama, 2010). Genistein and daidzein have been reported to stimulate cell growth at low concentration in ER-positive breast cancer cells (Li et al., 2008). Evidence from *in vivo* and *in vitro* studies indicates that anti-cancer activities of daidzein and genistein are mediated through cell cycle arrest and apoptosis (Jin et al., 2010; Kim, Lee, & Song, 2009; Li et al., 2008).

### **Cell Death Mechanisms**

Cell death can be classified according to morphological and enzymological criteria, functional aspects, or immunological characteristics. Recently, the Nomenclature Committee on Cell Death proposed unified criteria for the definition of cell death and its different morphologies (Kroemer et al., 2009). The most studied mechanisms of cell death are apoptosis, autophagy, and necrosis. Recently, the term necroptosis has been introduced to name a programmed necrosis (Zhivotovsky & Orrenius, 2010).

**Autophagy.** The processing and degradation of cytoplasmic components and organelles by the lysosome/vacuole are known as autophagy. It is characterized by formation autophagosomes, degradation of cytoplasmic contents, and slight chromatin condensation (Fink & Cookson, 2005). Under physiological conditions, autophagy is responsible for the elimination of defective proteins, injured or aged organelles, and

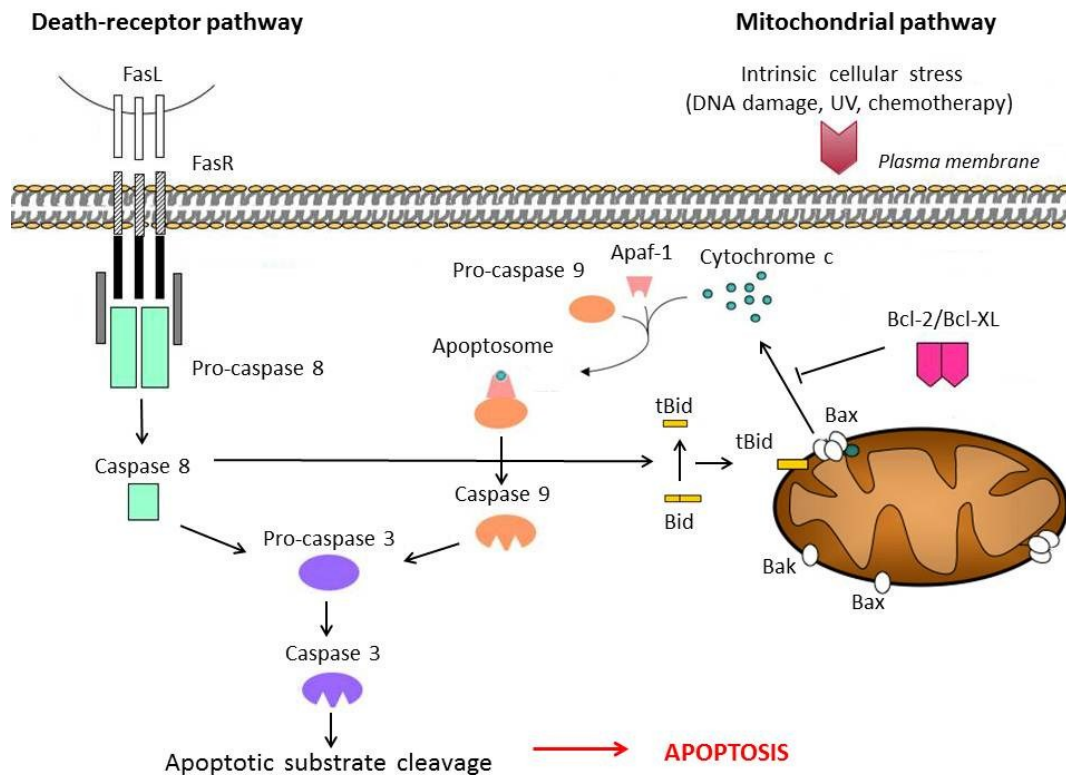
usually deregulated in cancers, neurodegenerative, and inflammatory disorders (Wong, Cheung, & Ip, 2011).

So far, at least 33 autophagy-related (*atg*) genes have been identified in yeast, and several homologues have been described in mammals (Li et al., 2012). The classical autophagic pathway acts through mTOR (mammalian target of rapamycin), a protein kinase that is important in cell growth, cell survival, and in negative regulation of autophagy (Duprez et al., 2009). mTOR, a serine/threonine protein kinase, is the major inhibitory signal that turns off autophagy in the presence of growth factors and nutrients. Class I PI3K is the key element controlling mTOR activation in response to insulin. Activated class I PI3K mainly uses phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P<sub>2</sub>) as a substrate to produce phosphatidylinositol (3,4,5)-triphosphate (PtdIns(3,4,5)P<sub>3</sub>) at the plasma membrane. PtdIns(3,4,5)P<sub>3</sub> increases membrane recruitment of PKB/Akt and its activator phosphoinositide-dependent protein kinase 1 leading to the activation of PKB/Akt. Activated PKB/Akt further activates mTOR through inhibiting the downstream protein complex Rheb. In the absence of nutrients and growth factors, mTOR is inhibited, leading to the induction of autophagy (Y. Li et al., 2012).

**Apoptosis.** Apoptosis is a form of programmed cell death associated with specific morphological features, such as cell shrinkage, plasma membrane blebbing, chromatin condensation, and formation of apoptotic bodies. B cell lymphoma 2 (Bcl-2) family proteins and caspases are key regulators of apoptosis (Duprez et al., 2009).

Apoptosis is divided into caspase-dependent (classical) and caspase-independent apoptosis (Constantinou, 2009). Radiation, growth factor depletion, or chemotherapeutic

drugs trigger the classical apoptotic cascade. Caspase-dependent apoptosis can be initiated by the extrinsic (death receptor-mediated) or the intrinsic (mitochondria-mediated) pathway (Sun & Peng, 2009) (Figure 3).



*Figure 3.* Schematic representations of extrinsic and intrinsic pathways of caspase activation. Death-receptor signaling involves direct caspase 8 mediated caspase 3 activation or Bid cleavage. Different apoptotic stimuli trigger the intrinsic mechanism and induce activation of Bax and Bak, leading to mitochondria permeabilization. Cytochrome c releases into cytosol and along with Apaf-1 and caspase 9 form the apoptosome. Active caspase 9 activates caspase 3, leading to apoptosis (Modified from (Bellance, Lestienne, & Rossignol, 2009)).

The extrinsic mechanism of apoptosis is induced by ligand binding to death receptors belonging to the tumor necrosis factor (TNF) receptor superfamily, such as TNFR, FasR, and TRAIL-R (Guicciardi & Gores, 2009). The death domain, an

intracellular portion of death receptor, functions as an adaptor protein. When the ligand binds to the receptor, it forms the death-inducing signaling complex (DISC). This complex then recruits the initiator caspases 8 and 10 and activates them. Activated caspases 8 and 10 are released into cytosol, leading to the activation of downstream executioner caspases 3, 6, and 7, which results in nuclear protein cleavage and initiation of apoptosis (Duprez et al., 2009; Kim et al., 2005; O'Brien, 2008).

Caspases are a family of intracellular cysteine proteases that cleave their substrates after Asp residue (Alenzi, Lotfy, & Wyse, 2010; Earnshaw, Martins, & Kaufmann, 1999; Li & Yuan, 2008). Caspases are divided into pro-inflammatory and pro-apoptotic. Pro-inflammatory caspases (caspase 1, 4, 5, 11, 12, 13, 14) are involved in cytokine maturation during the inflammatory response (Fink & Cookson, 2005). Pro-apoptotic caspases are further divided into initiator (caspase 2, 8, 9, 10) and executioner (caspase 3, 6, 7) caspases based on their role in apoptosis (Olsson & Zhivotovsky, 2011; Turk & Stoka, 2007). Initiator caspases with long prodomains, such as DED (death effector domain) and CARD (caspase recruitment domain), present in the cytosol as inactive monomers and are subsequently activated by oligomerization and proximity-induced autoproteolysis. Executioner caspases with short prodomains exist as dimers in the zymogen form and are activated by initiator caspases. Caspases are activated by being cleaved into a p20 large subunit and a p10 small subunit. The large subunit contains the catalytic dyad residues Cys and His, whereas the small subunit supplies several residues that form the substrate-binding groove (Li & Yuan, 2008; Pop & Salvesen, 2009; Turk & Stoka, 2007). Activated effector caspases selectively cleave a set



of target proteins to produce morphological and biochemical features associated with apoptosis (Fink & Cookson, 2005).

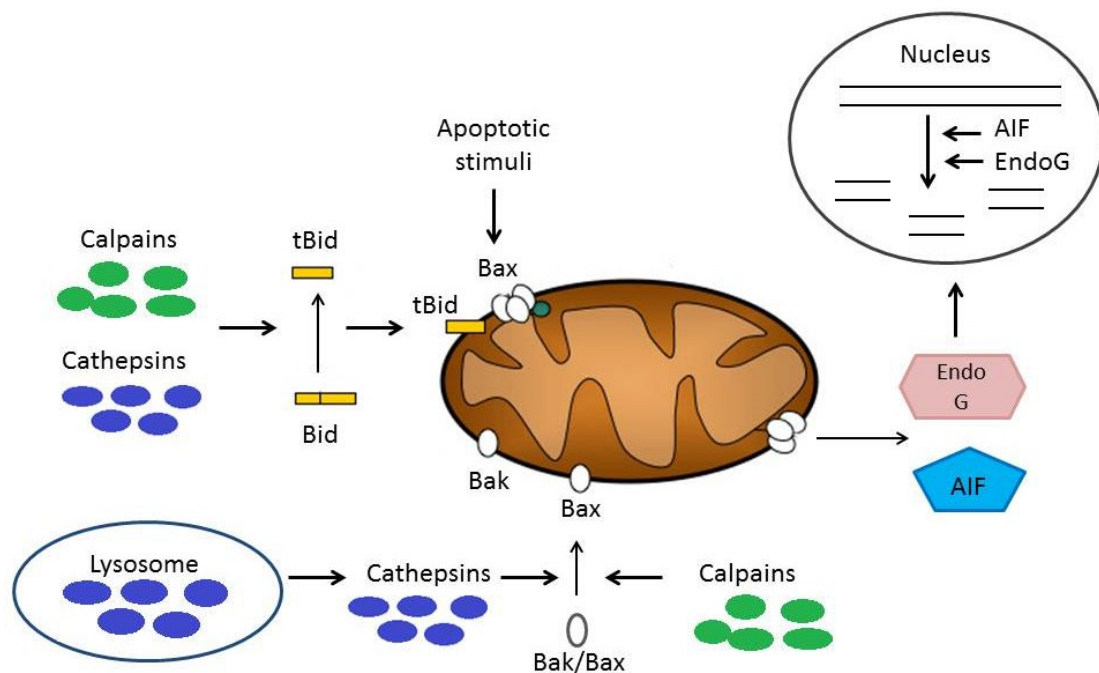
The intrinsic (mitochondrial) pathway is initiated as a response to cellular stress, such as DNA damage, oxidative stress, or cytotoxic agents. It is mediated by mitochondrial permeability transition, which is regulated by Bcl-2 family proteins (Galluzzi et al., 2012). The Bcl-2 protein family plays important roles in cell survival and cell death (Adams & Cory, 1998). At least 12 family members have been identified in mammalian cells. All members possess at least one of four conserved motifs known as Bcl-2 homology domains (BH1 to BH4) (Youle & Strasser, 2008). Bcl-2 proteins are grouped into two classes, anti-apoptotic and pro-apoptotic. Anti-apoptotic proteins (Bcl-2, Bcl-xL, Mcl-1, Bcl-w, A1/Bfl-1) have four BH domains and similar 3D structure. Overexpression of any one of these proteins is enough to inhibit pro-apoptotic Bcl-2 proteins and prevent apoptosis (Kelly & Strasser, 2011). The pro-apoptotic proteins Bax and Bak have three BH domains and are responsible for inducing mitochondria outer membrane permeabilization (MOMP), leading to the release of apoptotic proteins and caspase activation (Pradelli, Beneteau, & Ricci, 2010; Youle & Strasser, 2008). Bak and Bax appear to have overlapping function. Bak-deficient mice have no obvious defects, and those lacking Bax have a minor increase in spleen weight and male sterility. Bak/Bax double knockout mice have severe developmental defects (Kelly & Strasser, 2011).

BH3-only proteins (Bid, Bik, Bad, Hrk, Bim, Bmf, NOXA, PUMA) have a conserved BH3-domain and function as regulators of anti-apoptotic Bcl-2 proteins (Youle

& Strasser, 2008). BH3-only proteins trigger apoptosis by binding via their BH3 region to a groove on the surface of pro-survival proteins, thereby releasing Bax and Bak. They have also been reported to directly bind and activate Bax/Bak (Kelly & Strasser, 2011). In homeostasis, the anti-apoptotic Bcl-2 proteins inhibit activation of pro-apoptotic Bax/Bak. Under cellular stress, activated Bax and Bak oligomerize and form a pore complex on the outer mitochondrial membrane, leading to the release of cytochrome c, Smac/DIABLO, apoptosis inducing factor (AIF) and endonuclease G (EndoG). Cytochrome c with Apaf-1 and pro-caspase 9 form the apoptosome. Formation of the apoptosome results in the activation of caspase 9, which then activates caspase 3 and 7, leading to apoptosis (Constantinou, 2009; Duprez et al., 2009; O'Brien, 2008). The extrinsic apoptotic pathway is linked to the intrinsic pathway through caspase 8-mediated cleavage of Bid. Truncated Bid (tBid) also causes mitochondria outer membrane depolarization and release of apoptotic molecules (Duprez et al., 2009). Both extrinsic and intrinsic pathways merge at the level of activating the effector caspases. The effector caspases are responsible for cleavage of target proteins. One of these target proteins is poly-ADP-ribose polymerase (PARP). PARP catalyzes the poly ADP-ribosylation of a variety of nuclear proteins with NAD as substrate. Because it is activated by binding to DNA ends or strand breaks, PARP was suggested to contribute to cell death by depleting the cell of NAD and ATP. PARP was subsequently shown to be cleaved into 89 and 24 kDa fragments that contain the active site and the DNA-binding domain of the enzyme during drug-induced apoptosis. Such cleavage essentially inactivates the enzyme by

destroying its ability to respond to DNA strand breaks (Boulares et al., 1999; Herceg & Wang, 1999).

Mitochondria are not only responsible for caspase-dependent apoptosis, but also for caspase-independent programmed cell death (CI-PCD) (Figure 4). Up-regulation of pro-apoptotic Bak/Bax is a requirement for initiation of CI-PCD. In response to apoptotic stimuli, cathepsins and calpains cleave Bid and activate Bak and Bax (Constantinou, 2009). Cathepsins are lysosomal proteins belonging to three classes of enzymes: serine, aspartic, and cysteine proteases. They are synthesized as inactive pro-enzymes and activated within the acidic environment of the lysosome.



*Figure 4.* Model of caspase-independent programmed cell death. Activated cathepsins and calpains are responsible for the activation of Bax/Bak, cleavage of Bid and AIF. Presence of Bax/Bak and tBid at the mitochondrial membrane cause its depolarization and release of AIF, EndoG, and Smac/DIABLO. AIF along with EndoG translocate to the nucleus, where they induce chromatin condensation and high molecular weight DNA fragmentation.

Calpains are non-lysosomal cysteine proteases present in the cytosol and activated by  $\text{Ca}^{2+}$  influx into the cell caused by endoplasmic reticulum stress (Norberg, Orrenius, & Zhivotovsky, 2010). Activated by cathepsins and calpains, pro-apoptotic tBid, Bax, and Bak translocate to mitochondria causing their permeabilization and release of AIF, EndoG, Smac/DIABLO, and HtrA2/Omi (Broker, Krut, & Giaccone, 2005; Guicciardi, Leist, & Gores, 2004; Tait, 2008). AIF, a 62 kDa protein, is anchored to the internal mitochondrial membrane, where it oxidizes NADH (Zhivotovsky & Orrenius, 2010). Prior to the release, AIF must be liberated from its membrane anchor. Several studies show that cathepsins and calpains are also responsible for AIF proteolysis (Norberg et al., 2010). Two main isoforms of calpains, calpain-I and calpain-II, co-exist with the specific inhibitor calpastatin. Calpastatin prevents calpain activation. Generally, calpains are cytosolic enzymes, but calpain-I is present in mitochondrial intermembrane space and is considered to be the main enzyme responsible for cleavage of AIF. Some studies suggest that only cysteine cathepsins (cathepsin B, L and S) are able to cleave AIF (Norberg et al., 2010). Endonuclease G is located in the mitochondrial intermembrane space and is involved in regulating mitochondrial biogenesis, DNA synthesis, and repair (Tait, 2008). After release from mitochondria, AIF and EndoG translocate to the nucleus, where they induce chromatin condensation and high molecular weight (~50 kb) DNA fragmentation (Constantinou, 2009).

**Programmed Necrosis (Necroptosis).** For a long time, necrosis has been regarded as an accidental and uncontrolled form of cell death. However, accumulating evidence revealed that necrosis can occur in a regulated manner (Galluzzi & Kroemer,

2008). Programmed necrosis, or necroptosis, is characterized by cell and organelle swelling, formation of intracellular vacuoles, and loss of cell membrane integrity (Challa & Chan, 2010). Regulated necrosis can occur during mammalian development as well as in adult tissue homeostasis. Necroptosis is involved in several pathological conditions, such as neurodegeneration, ischemia-reperfusion, and infection. Necrosis can function as a back-up cell death mechanism when apoptosis is disabled (Galluzzi et al., 2011).

Necroptosis can be induced by the ligation of TNF superfamily death receptors and requires activity of serine/threonine kinases RIP1 and RIP3. Formation of a “pro-necrotic complex” by RIP1 and RIP3 and its subsequent phosphorylation initiates necrotic events. During death-receptor-induced apoptosis, RIP1 and RIP3 are cleaved by caspase 8 and become non-functional. Necroptosis can also be initiated by pathogen recognition receptors, including transmembrane toll-like receptors (TLRs), the cytosolic NOD-like receptors (NLRs), and the RIG-I-like receptors (RLRs). These receptors are expressed by cells of the innate immunity to sense pathogen-associated molecular patterns, such as viral or bacterial nucleotides, lipoproteins, or dsRNA and respond by triggering inflammation or cell death (Challa & Chan, 2010; Duprez et al., 2009; Vandenabeele, Galluzzi, Vanden Berghe, & Kroemer, 2010). An execution phase of necroptosis occurs with the help of reactive oxygen species, cathepsins, calpains, calcium, and ceramides (Duprez et al., 2009). Massive DNA damage activates PARP-1, which stimulates the mitochondrial release of AIF. Depletion of cytosolic ATP by PARP-1 overactivation causes AIF translocation to the nucleus, where it mediates DNA degradation and cell death (Vandenabeele et al., 2010). In addition, PARP-2 was

described as one of the core regulators of necroptosis (Galluzzi & Kroemer, 2008; Hitomi et al., 2008). PARP-1 and PARP-2 possess overlapping and non-redundant functions in the maintenance of genomic stability (Menissier de Murcia et al., 2003).

Previous findings from our lab indicate that flavonoids are able to induce cytotoxicity in cancer cells and inhibit caspase activity (White et al., 2012). These results suggest that classical apoptosis is not the primary form of flavonoid-induced cell death. Our goal was to determine whether flavonoids induce cell death through caspase-dependent or caspase-independent mechanism.

## **Method**

### **Materials**

Flavonoids were purchased from Alexis Biochemicals, Indofine Chemicals, or Sigma. Flavonoids were dissolved in 100% dimethyl sulfoxide (DMSO) to yield a 50 mM stock solution. Primary and secondary antibodies were purchased from Cell Signaling Technology and Santa Cruz Biotechnology.

### **Cell Culture**

The MDA-MB-231 (human breast adenocarcinoma) cell line was obtained from American Type Culture Collection (ATCC). Caspase 3/7-deficient mouse embryonic fibroblasts (MEFs) and wild-type control cell lines were a kind gift of Richard Flavell; baby mouse kidney (BMK) cells were a gift from Ellen White. Cells were grown at 37°C under humidified 5% CO<sub>2</sub> conditions in Dulbecco's Modification of Eagle's Medium (DMEM) with high glucose, L-glutamine, sodium pyruvate (Cellgro), supplemented with 10% Fetal Bovine Serum (FBS) and 1% antibiotic. MDA-MB-231 cells were maintained as recommended by ATCC. Caspase 3/7 deficient MEFs and BMK cells were used for no more than five passages.

### **Quantitative Measurement of Cytochrome c Release**

Quantification of cytochrome c release was performed by flow cytometry. Approximately  $3.0 \times 10^6$  MDA-MB-231 cells were seeded in 10 cm culture dishes (BD Biosciences), grown overnight, and then incubated with indicated concentrations of

flavonoid for 24, 36, and 48 hr. Collected cells were permeabilized with digitonin (0.5 mg/ml) in Phosphate Buffered Saline (PBS) with 100 mM KCl for 10 min on ice and fixed with 4% paraformaldehyde for 20 min. Cells were pelleted and incubated in blocking buffer (0.05% saponin, 3% BSA in PBS) for 30 min on ice. After washing, cells were incubated with anti-cytochrome c antibody at a dilution of 1/250 (Santa Cruz Biotechnology) in blocking buffer overnight at 4<sup>0</sup>C, and then for 30 min with Alexa Fluor 633-conjugated secondary antibody (Invitrogen) before analysis using FACScalibur (BD Bioscience). We regarded the cells with low fluorescence as having low levels of cytochrome c and those with high fluorescence as having intact mitochondria.

### **Cell Lysates Preparation**

Approximately  $3.0 \times 10^6$  of MDA-MB-231 cells were plated on 10 cm culture dishes, grown overnight, and then treated with 100  $\mu$ M flavonoid for 0, 1, 2, 3, 4, 5, 6, 24, and 48 hr. At each time point, cells were lysed in lysis buffer (50 mM Tris-Cl pH 7.5, 300 mM NaCl, 0.5% sodium deoxycholate, 5 mM EDTA, 1% NP-40, 1% SDS) along with 1X Halt protease inhibitor cocktail (Thermo Scientific). Cell lysates were incubated on ice for 30 min, then sonicated five times for 5 s and centrifuged at 12,000 rpm for 10 min to remove cell debris. Protein concentration was measured using Pierce BCA Protein Assay Kit (Thermo Scientific) following manufacturer's recommended protocol. Cell lysates were stored at -80<sup>0</sup>C.



## **Sodium Dodecylsulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)**

1.5 mm 7% SDS-PAGE gel ([running gel: 5.75 ml of MilliQ H<sub>2</sub>O, 2.5 ml of 4X lower buffer (375 ml of 2M Tris at pH 8.8, 10 ml of 10% SDS, 115 ml MilliQ H<sub>2</sub>O), 1.75 ml of 40% acrylamide, 60 µl of 10% APS and 15 µl of TEMED]; [stacking gel: 3.225 ml of MilliQ H<sub>2</sub>O, 1.25 ml of upper buffer (125 ml of 2M Tris at pH 6.8, 10 ml of 10% SDS, 365 ml of MilliQ H<sub>2</sub>O), 525 µl of 40% acrylamide, 25 µl of APS and 6 µl of TEMED]) or 12% gel (running gel: 4.5 ml of MilliQ H<sub>2</sub>O, 2.5 ml of 4X lower buffer (375 ml of 2M Tris at pH 8.8, 10 ml of 10% SDS, 115 ml MilliQ H<sub>2</sub>O), 3 ml of 40% acrylamide, 60 µl of 10% APS and 15 µl of TEMED]) were used to separate proteins for western blotting.

## **Western Blotting**

Protein samples were mixed with 2X Laemmli buffer (125 mM Tris HCl pH 6.8, 20% glycerol, 4% SDS, 10% 2-mercaptoethanol, and 0.004% bromphenol blue) and heated at 95<sup>0</sup>C for 5 min. Samples of 100 µg total protein were loaded on 7% or 12% SDS-PAGE and then electrophoretically transferred to PVDF membrane (Millipore) using 1X transfer buffer (25 mM Tris and 192 mM Glycine, pH 8.3) at 250 mA for 1 hr. Membranes were blocked with 2% ECL Advance blocking agent (GE Healthcare), dissolved in 0.1% TBS-Tween 20 (50 mM NaCl, 20 M Tris HCl pH 7.6, 0.1% Tween 20), for 1 hr at room temperature and incubated with primary antibody at 4<sup>0</sup>C overnight. The following antibodies and dilutions were used in western blots: anti-cleaved caspase 3 (17, 19 kDa) at 1/1000 concentration, anti-PARP (116, 89 kDa) at 1/1000 concentration

(Cell Signaling Technology), and anti-GAPDH (37 kDa) at 1/2000 concentration (Santa Cruz Biotechnology). Membranes were washed with TBS-Tween (3 times - 5 min per wash) and probed with secondary horseradish peroxidase (HRP) conjugated goat anti-rabbit antibody (Cell Signaling Technology) for 1 h at room temperature. Membranes were developed using the Amersham ECL Advance or ECL Prime Western Blotting Detection Reagent (GE Healthcare) according to the manufacturer's suggested protocol. Proteins were detected by chemiluminescence using the ImageQuant Las4000 imager system (GE Healthcare).

### **Trypan Blue Viability Test**

$2.5 \times 10^4$  BMK cells were seeded into 6-well plate and treated with 100  $\mu$ M flavonoid for 72 hr. Cells were trypsinized, washed, and resuspended in 1 ml of 1X PBS (100 ml 10X PBS and 900 ml MilliQ H<sub>2</sub>O). Cell suspension was mixed with equal volume of trypan blue and counted using TC10 Automated cell counter (Bio-Rad).  $3 \times 10^6$  wild-type and caspase 3/7 deficient MEFs were plated into 10 cm culture dishes, then treated with 100  $\mu$ M flavonoid for 72 hr, and counted with hemocytometer.

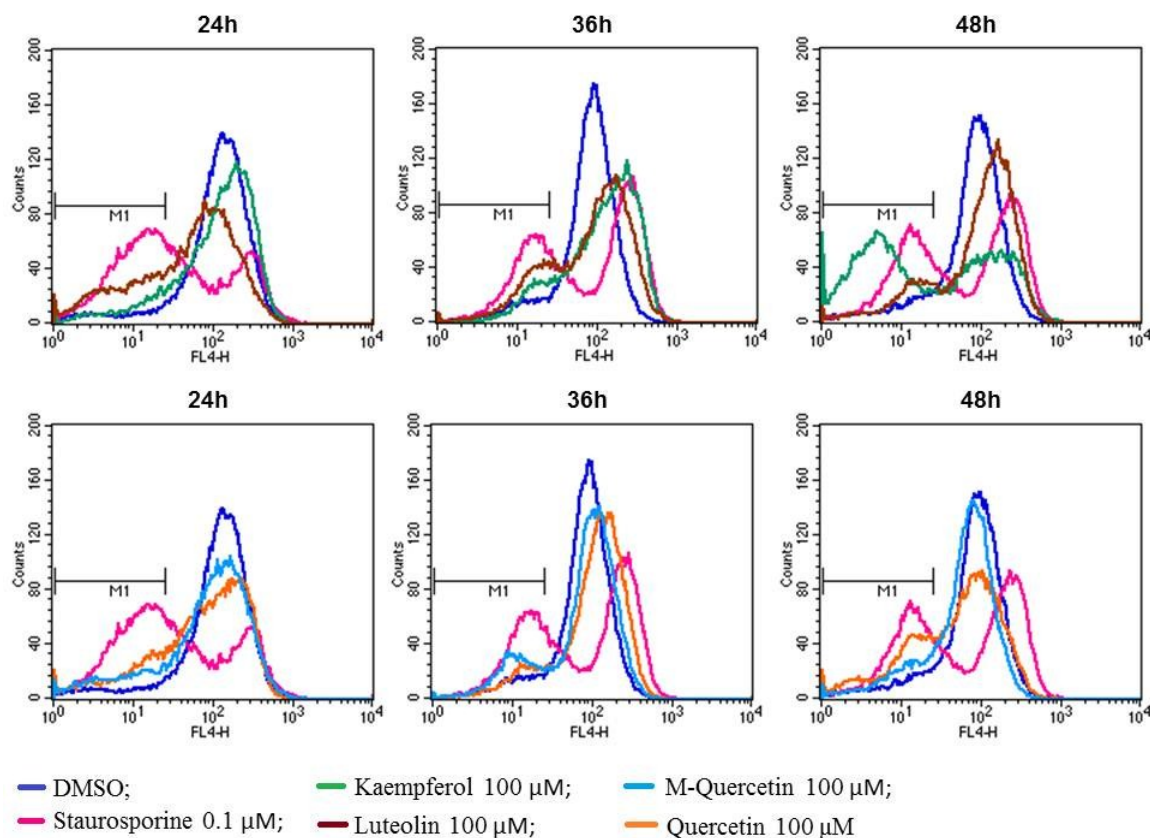
### **Statistical Analysis**

For comparisons between two groups, the data were analyzed using two-tailed Student *t*-test. A *p* value <0.05 was regarded as statistically significant.

## Results

### **MDA-MB-231 cells Release Cytochrome c after Flavonoid Treatment**

Intracellular cytochrome c levels were evaluated by means of flow cytometry. MDA-MB-231 breast cancer cells were treated with kaempferol, quercetin, luteolin, and methyl-quercetin at 100  $\mu$ M and harvested at 24, 36, and 48 hr. Cells were prepared for flow cytometric acquisition as described in the Method section. DMSO treated cells were used as negative control, 0.1  $\mu$ M staurosporine (STR) and 200  $\mu$ M cycloheximide were used as positive controls. Staurosporine is a non-selective protein kinase inhibitor that is often used as a general method for inducing apoptosis (Kabir, Lobo, & Zachary, 2002). Cycloheximide inhibits protein synthesis in eukaryotes by interfering with the translocation step, thus blocking translational elongation (Schneider-Poetsch et al., 2010). Cytochrome c under normal conditions is localized in mitochondria. Following treatment with an apoptotic stimulus, such as staurosporine, cytochrome c will be released to the cytoplasm. Flow cytometric analysis showed that flavonoids induce cytochrome c release in a time-dependent manner (Figure 5). During analysis, marker 1 (M1) was set to quantitate the percentage of cells that released cytochrome c (cells with low fluorescence). Maximum cytochrome c release was seen after 48 hr of incubation after kaempferol and quercetin treatment, but luteolin and methyl-quercetin treatment showed the highest percentage of dead cells at 36 hr (Table 1).



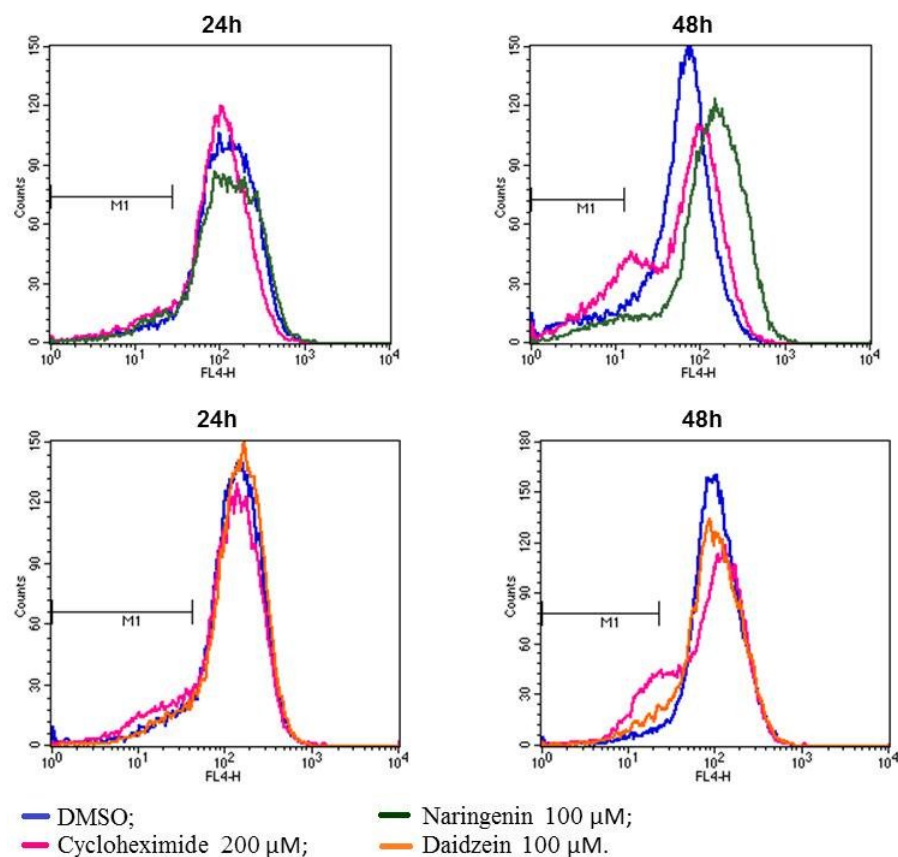
*Figure 5.* Cytochrome c release from MDA-MB-231 cells after flavonoid treatment. The number of cells that released cytochrome c (cells with low fluorescence) was slightly increased at 24 hr compared to DMSO. The number of dead cells was increased more after 36 and 48 hr of exposure. The number of live cells after flavonoid treatment significantly decreased compared to the negative control.

Table 1

*Percentage of Cytochrome c Released Cells after Flavonoid Treatment*

Flavonoids	% of dead cells		
	24hr	36hr	48hr
DMSO	7.38	10.86	11.25
Staurosporine 0.1 $\mu$ M	65.8	49.03	45.16
Kaempferol 100 $\mu$ M	9.64	13.58	53.64
Luteolin 100 $\mu$ M	25.29	27.56	16.29
Quercetin 100 $\mu$ M	19.55	17.23	29.36
M-Quercetin 100 $\mu$ M	15.14	19.69	11.81

Previous studies on flavonoids in our lab have shown that the flavonoids naringenin, methyl-naringenin, catechin, myricetin, daidzein, and genistein are weakly or non-cytotoxic to breast cancer cells. To determine if these flavonoids cause cytochrome c release, flow cytometric analysis was performed using MDA-MB-231 cells after 24 and 48 hr exposure with daidzein and naringenin at 100  $\mu$ M. Our results confirmed previous findings and showed that naringenin and daidzein do not induce the release of cytochrome c (Figure 6).



*Figure 6.* Naringenin and daidzein do not induce cytochrome c release in MDA-MB-231 breast cancer cells. The number of cells that released cytochrome c after treatment with naringenin and daidzein was similar to DMSO treatment at both 24 and 48 hr.

Our results showed that the percentage of cytochrome c released cells after treatment with daidzein and naringenin was similar to the negative control (Table 2 and 3).

Table 2

*Percentage of Cytochrome c Released Cells after Daidzein Treatment*

Flavonoids	% of dead cells	
	24hr	48hr
DMSO	12.66	7.98
Cycloheximide 200 $\mu$ M	20.6	31.17
Daidzein 100 $\mu$ M	10.95	14.38

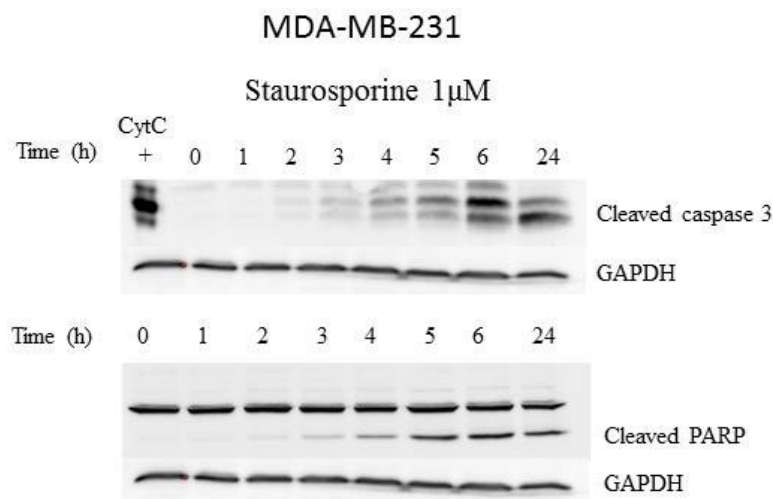
Table 3

*Percentage of Cytochrome c Released Cells after Naringenin Treatment*

Flavonoids	% of dead cells	
	24hr	48hr
DMSO	13.18	15.72
Cycloheximide 200 $\mu$ M	18.54	33.83
Naringenin 100 $\mu$ M	17.12	13.5

### **Activation of Caspase 3 and PARP Cleavage was Detected in MDA-MB-231 Cells after Treatment with some Flavonoids**

To understand the mechanism of flavonoid cytotoxicity, western blot analysis was performed to identify the main apoptotic proteins, cleaved caspase 3, and cleaved PARP. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control to confirm that the protein loading was the same in all lanes of the gel. Staurosporine-treated MDA-MB-231 cells showed early activation of caspase 3 and PARP cleavage and were used as a positive control (Figure 7).

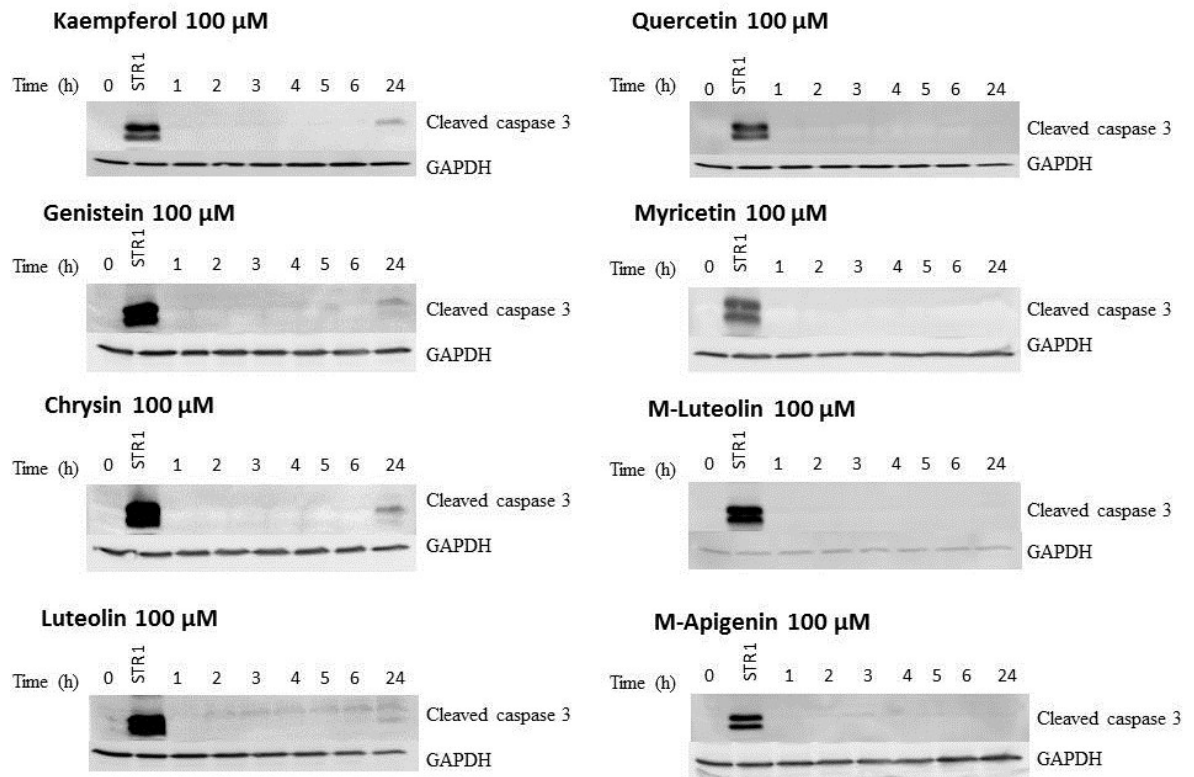


*Figure 7.* Caspase 3 activation and PARP cleavage in staurosporine-treated MDA-MB-231 cells. Cells were treated with 1  $\mu$ M Staurosporine and protein extracts prepared at the time as indicated. Earliest identification of cleavage of caspase 3 and PARP was seen after 2 hr of incubation with maximum band intensity at 6 and 24 hr. The data were representative of three independent experiments. CytC – Cytochrome c Treated Jurkat Cell Extracts (Cell Signaling, Cat# 9663).

Flavonoid-treated MDA-MB-231 cells were collected at various time points, and lysates of 100  $\mu$ g of total protein were resolved on SDS-PAGE followed by western blotting, as described in the Method section. Staurosporine-treated cell lysates were used as a positive control. The western blot analysis results showed activation of caspase 3 and PARP cleavage after treatment with some flavonoids (Figure 8A and B).

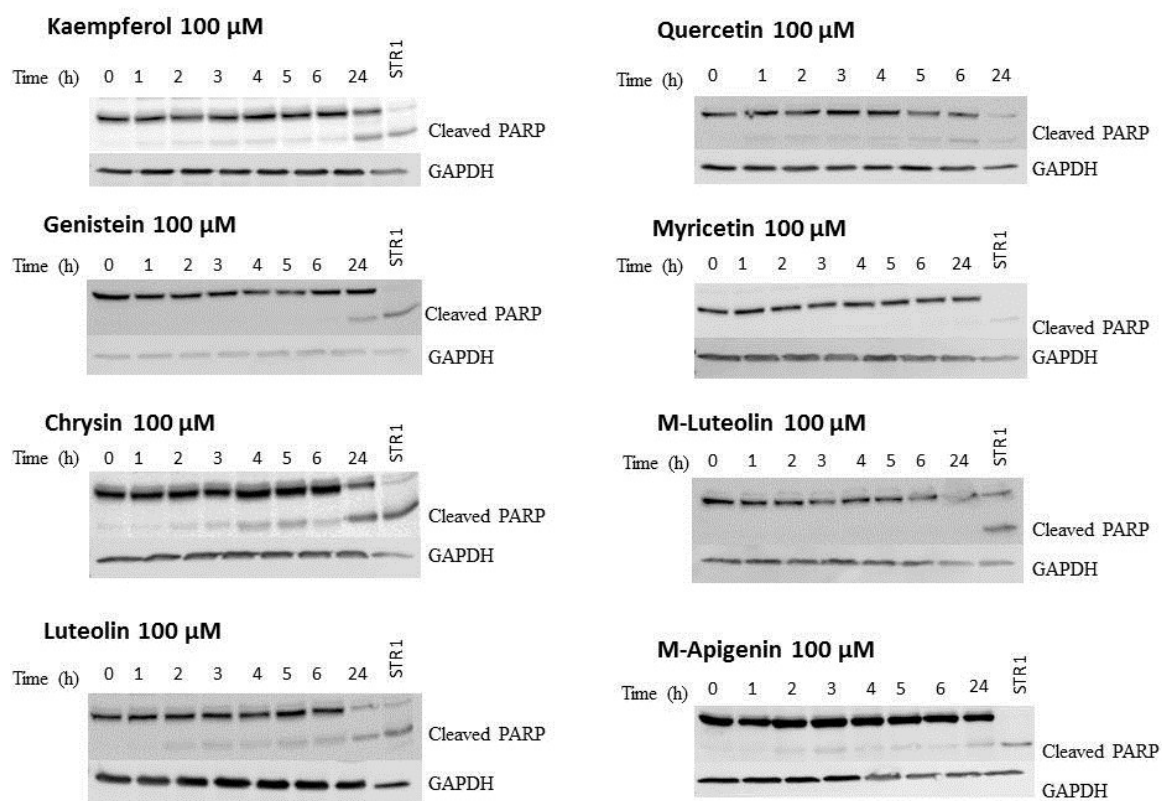
Kaempferol and chrysin treatment showed caspase 3 activation at 24 hr and PARP cleavage after 1 hr of incubation with maximum band intensity at 24 hr. We were able to detect caspase 3 and PARP cleavage at 24 hr after incubation with genistein. Luteolin treatment showed PARP cleavage and activation of caspase 3 after 2 hr of incubation. We did not observe caspase 3 cleavage, but we were able to detect PARP cleavage

starting at 2 hr after treatment with quercetin and methyl-apigenin. Myricetin and methyl-luteolin showed neither caspase 3 nor PARP cleavage at any time points (Figure 8A and B).



*Figure 8A.* Cleaved caspase 3 was detected after some but not all flavonoid treatments. Results showed cleavage of caspase 3 after 24 hr of treatment with kaempferol, genistein, and chrysin. Earliest activation of caspase 3 was detected at 2 hr after luteolin treatment. Caspase 3 activation was not detected after incubation with quercetin, myricetin, m-luteolin, and m-apigenin. The data were representative of three independent experiments.



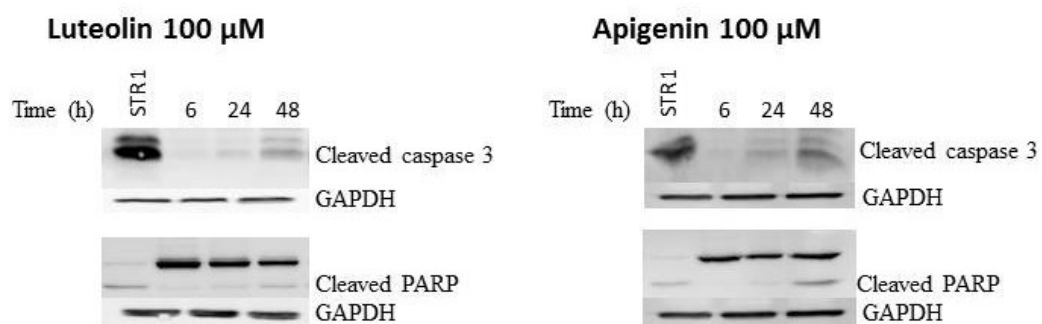


*Figure 8B.* PARP cleavage was detected after treatment with some flavonoids. PARP cleavage corresponded to the caspase 3 activation after treatment with genistein. Cleavage of PARP was observed earlier than cleavage of caspase 3 after treatment with kaempferol, chrysin, and luteolin. Myricetin and m-luteolin treatment showed no PARP cleavage. The data were representative of three independent experiments.

### **Some Flavonoids do not Induce Caspase 3 Activation and PARP Cleavage even at Later Time Points**

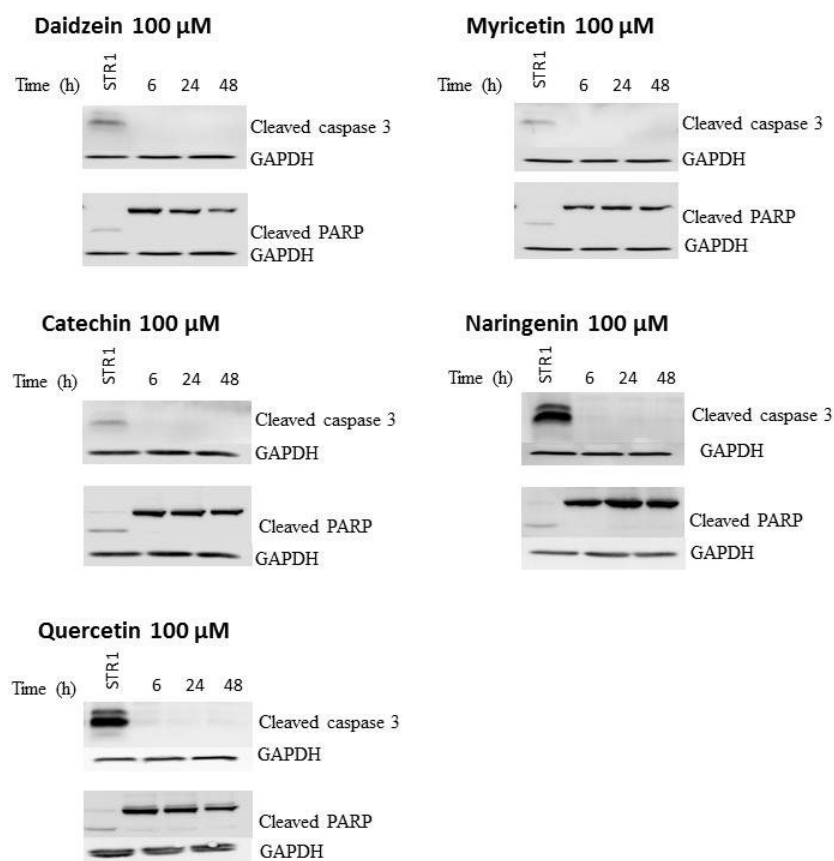
Figure 8 shows the time-dependent activation of caspase 3 and cleavage of PARP. Some flavonoids did not induce activation of the main apoptotic markers at the 24 hr time point. They may induce cell death later than 24 hr. To determine if flavonoids trigger caspase 3 activation and PARP cleavage at a later time, we collected cell lysates at 6, 24 and 48 hr and performed western blotting, as described in the Method section. Previous

data from our lab showed that apigenin and luteolin were highly-cytotoxic to breast cancer cells. Our results showed that apigenin and luteolin treatment induced caspase 3 and PARP cleavage with maximum band intensity at 48 hr (Figure 9).



*Figure 9.* Caspase 3 activation and PARP cleavage in MDA-MB-231 cells after flavonoid treatment. Results showed that increase of cleaved caspase 3 and PARP were detected at later time points after treatment with apigenin and luteolin. The data were representative of three independent experiments.

Daidzein, catechin, myricetin, and naringenin have been shown to have a weak or non-cytotoxic effect on breast cancer cells (data not shown). The cleavage of caspase 3 and PARP was not detected after incubation with myricetin, catechin, daidzein, and naringenin (Figure 10). PARP cleavage, but not caspase 3 activation, was observed after quercetin treatment, which corresponds to our previous results shown in Figure 8A and B.

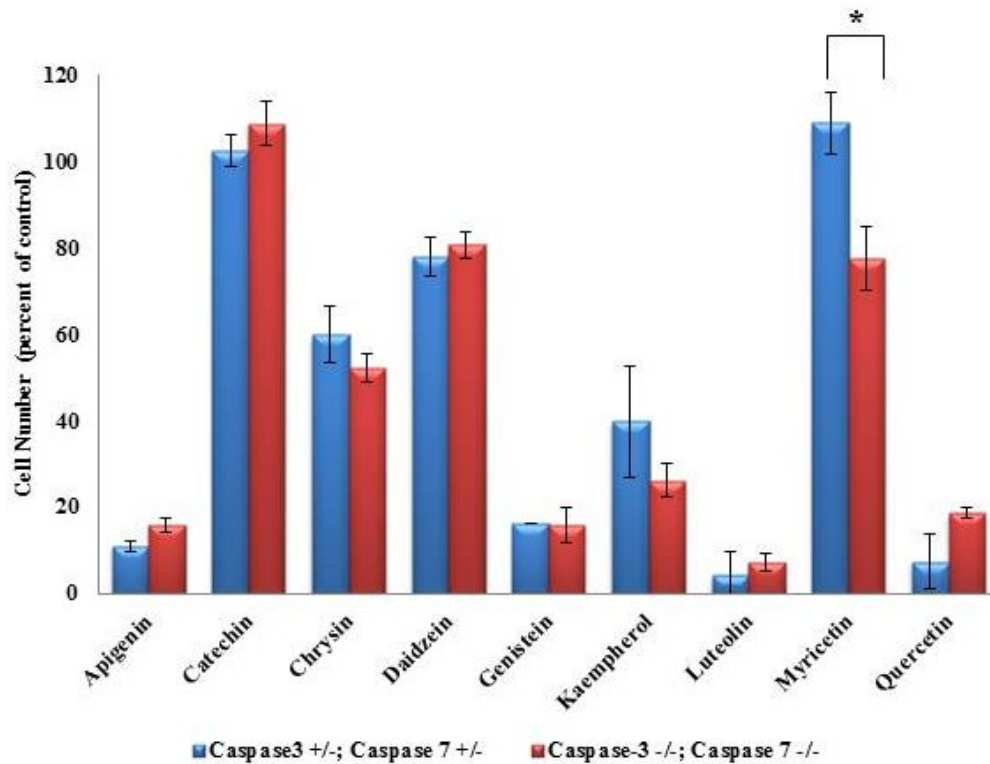


*Figure 10.* Some flavonoids do not induce cleavage of caspase 3 and PARP. Cleaved caspase 3 and PARP were not detected after treatment with daidzein, myricetin, catechin, and naringenin. Quercetin treatment showed PARP cleavage, but not activation of caspase 3. The data were representative of three independent experiments.

### Flavonoid Induced Cell Death does not Require Caspases

To determine whether caspases are main factors in flavonoid-induced cell death, we used caspase 3/7 knockout cells. A cell viability test was performed on MEF 119 (Caspase 3<sup>-/-</sup>/Caspase 7<sup>-/-</sup>) and MEF 122 (Caspase 3<sup>+/-</sup>/Caspase 7<sup>+/-</sup>, wild-type) cells after 72 hr of 100  $\mu$ M flavonoid treatment. There was no significant difference observed in the cytotoxicity of flavonoids tested on caspase 3/7 double knockout cells compared to wild-

type (Figure 11), except for myricetin, which was slightly cytotoxic to the caspase deficient MEFs ( $p < 0.05$ ).

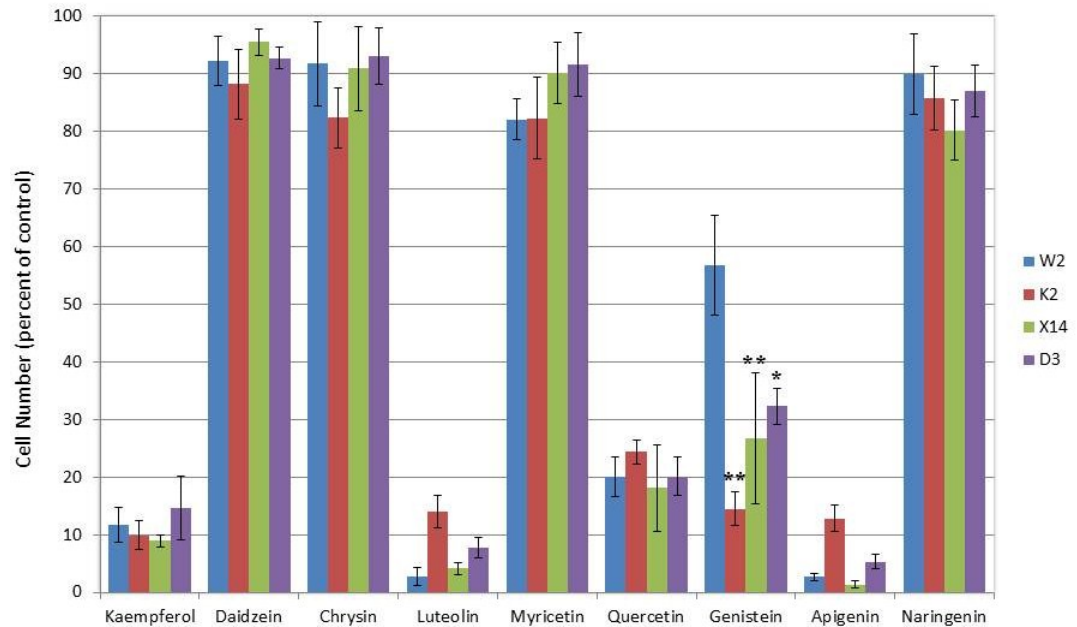


*Figure 11.* Percent viability of caspase-deficient MEFs compared to the wild-type after flavonoid treatment. Results showed no significant difference between caspase deficient and caspase containing MEFs suggesting that caspase 3 and 7 are not required for flavonoid-induced cell death. Error bars represent standard deviation from three independent experiments. \* $p < 0.05$ , t-test.

### **Pro-Apoptotic Bak and Bax are not Essential for Flavonoid-Induced Cell Death**

Bak and Bax are pro-apoptotic proteins that belong to a Bcl-2 family and are responsible for mitochondrial outer membrane permeabilization (Youle & Strasser, 2008). To determine if Bak and Bax are required for flavonoid-induced cell death, a trypan blue viability test was performed on wild-type (W2), Bak (K2), Bax (X14) and Bak/Bax (D3)-deficient BMK cells after treatment with 100  $\mu$ M flavonoids for 72 hr. No

significant difference was observed in viability of Bak/Bax deficient BMK cells compared to wild-type, suggesting that flavonoid-induced cytotoxicity does not require Bak/Bax activation. Only genistein treatment showed weak cytotoxicity to Bak/Bax single and double knockout cells ( $p < 0.05$ ,  $p < 0.0005$ , respectively) (Figure 12).

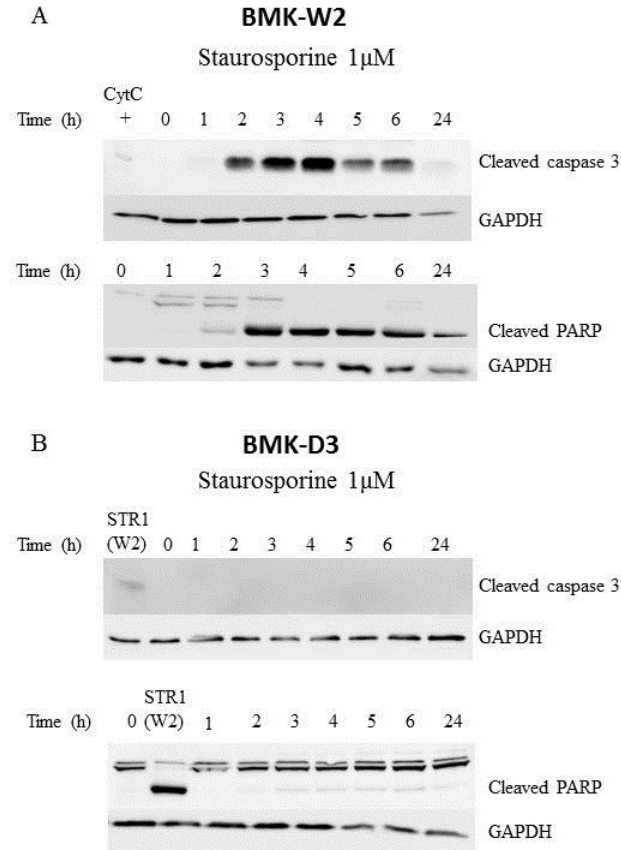


*Figure 12.* Flavonoid-induced cell death does not require Bak/Bax activation. Cellular number as a percent of DMSO-treated cells after 72 hr of flavonoid treatment. Results showed no significant difference in flavonoid cytotoxicity in Bak/Bax deficient BMKs compared to wild-type. Error bars represent standard deviation from three independent experiments. \* $p < 0.05$ , \*\* $p < 0.0005$ , t-test.

### Caspase 3 Activation was not Observed in Bak/Bax Double Knockout Cells

To better understand the mechanism of flavonoid-induced cytotoxicity, western blot analysis was performed in Bak/Bax deficient cells (BMK-D3) against cleaved caspase 3 and PARP. We were able to detect caspase 3 activation and PARP cleavage in wild-type (BMK-W2) cells after 2 hr of incubation with staurosporine (Figure 13A). We

did not observe cleaved caspase 3 in Bak/Bak double knockout cells, but detected PARP cleavage after 3 hr of incubation with staurosporine (Figure 13B).



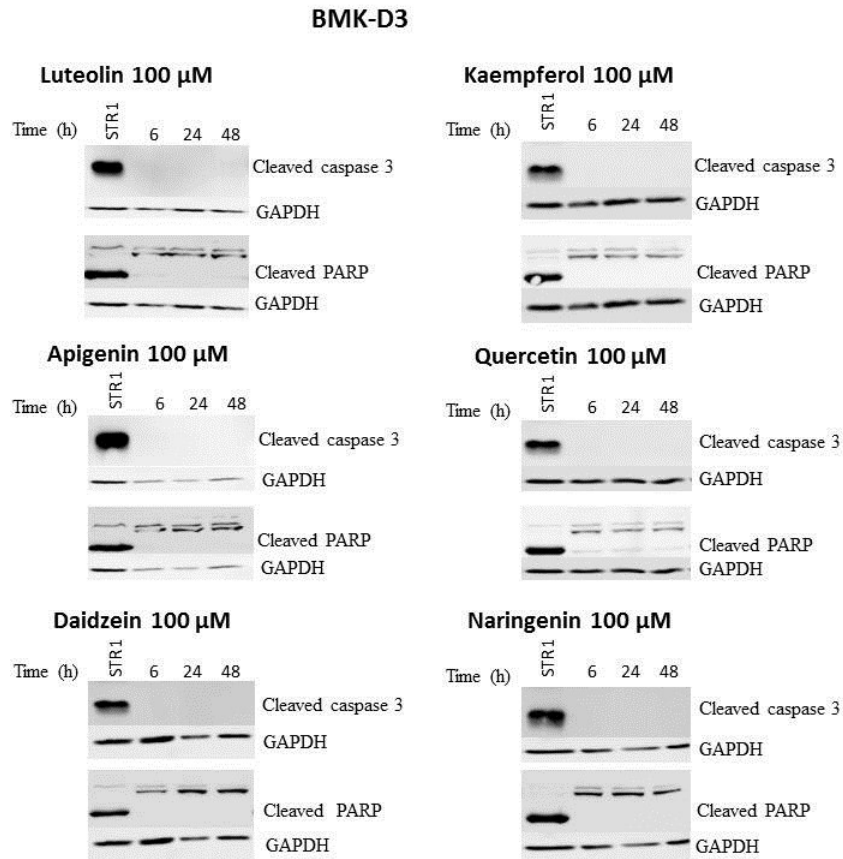
*Figure 13.* Cleaved caspase 3 and PARP detection in wild-type (BMK-W2) and Bak/Bax deficient (BMK-D3) cells after staurosporine treatment. Cells were treated with 1 μM staurosporine and protein extracts were prepared at the time as indicated. The data were representative of three independent experiments.

- A. Earliest identification of caspase 3 and PARP cleavage in BMK-W2 cells was seen after 2 hr of incubation with maximum band intensity at 3 and 4 hr.
- B. No caspase 3 activation was observed in BMK-D3 cells. Cleavage of PARP was detected after 3 hr of incubation with STR.

BMK-D3 cells were treated with 100 μM flavonoid and collected at 6, 24, 48 hr time points. Our results showed that neither caspase 3 activation, nor PARP cleavage

was observed in BMK-D3 cells after incubation with flavonoids, except for quercetin.

PARP cleavage was detected only after quercetin treatment (Figure 14).



*Figure 14.* No caspase 3 activation and PARP cleavage was observed in BMK-D3 cells after flavonoid treatment. PARP cleavage was detected only after quercetin treatment. The data were representative of three independent experiments.

## Discussion

### Flavonoids Trigger Cytochrome c Release in MDA-MB-231 Breast Cancer Cells

Activation of the apoptotic pathway is a key mechanism by which anticancer drugs kill tumor cells. Flavonoids have been reported to induce extrinsic and intrinsic apoptosis in breast cancer cells (Choi & Kim, 2009; Jin et al., 2010; Shanmugaraj et al., 2010). Mitochondria play an important role in the apoptotic process. Cytochrome c and other pro-apoptotic factors release into the cytosol after MOMP (Duprez et al., 2009). We found that flavonoids, such as kaempferol, luteolin, quercetin, and methyl-quercetin induced cytochrome c release in a time-dependent manner (Figure 5). Several studies have reported that flavonoids induce mitochondrial dysfunction leading to cytochrome c release (Table 4).

Table 4

*Flavonoids that have been Reported to Induce Cytochrome c Release in Different Cell Lines*

Flavonoids	Cell line	Reference
Acacetin	MCF-7 breast cancer	(Shim et al., 2007)
Apigenin	MDA-MB-453 breast cancer	(Choi & Kim, 2009)
Baicalein	MDA-MB-231 breast cancer	(Lee et al., 2008)
Cajanol	MCF-7 breast cancer	(Luo et al., 2010)
Chrysin	U937 leukemia	(Woo et al., 2004)
Daidzein	MCF-7 breast cancer	(Jin et al., 2010)
EGCG	Hep2 epidermoid carcinoma	(Lee et al., 2010)
Eupafolin	HeLa cervical adenocarcinoma	(Chung, 2010)
Jaceosidin	CAOV-3 ovary cancer	(Lv, Sheng, Chen, Xu, & Xie, 2008)
Liquiritigenin	HeLa cervical adenocarcinoma	(Liu et al., 2011)
Quercetin	MDA-MB-231 breast cancer	(Chien, 2009)



In the cytosol, cytochrome c activates caspase 9 leading to downstream caspase activation (Choi & Kim, 2009; Gogada et al., 2011). We found that daidzein and naringenin did not cause cytochrome c release (Figure 6). Based on our data, we suggest that flavonoids such as catechin, myricetin, naringenin, methyl-naringenin, and daidzein are weakly or non-cytotoxic to breast cancer cells (data not shown). Our findings differ from another report found on daidzein and its ability to induce apoptosis in MCF-7 breast cancer cell line through cytochrome c release and activation of caspase cascade (Jin et al., 2010). Cytochrome c release does not imply that cell death after flavonoid treatment occurred through caspase-dependent mechanism; therefore, we examined the activation of caspase 3 and PARP cleavage.

### **Flavonoid-Induced Cell Death Might be Caused by Caspase-Independent Mechanism**

The transduction and execution of apoptotic signals require coordinated activation of caspases. One of the critical enzymes is caspase 3. Caspase 3 is activated and responsible for cleavage and inactivation of other proteins, such as PARP or inhibitor of caspase-activated DNase (ICAD) (Kitazumi & Tsukahara, 2011). In order to further understand the molecular mechanism involved in apoptosis caused by flavonoids, the expression of the apoptosis-related proteins, such as cleaved caspase 3 and PARP, were assessed in MDA-MB-231 cells.

We found that flavonoids such as kaempferol, genistein, chrysin, luteolin, and apigenin induced caspase 3 activation and PARP cleavage (Figure 8 and 9). Kaempferol has been reported to activate caspase 7 and PARP in the caspase 3 deficient breast cancer

cell line MCF-7 (Kang et al., 2009). Kim et al. (2009) demonstrated that genistein induced apoptosis through caspase-dependent extrinsic and intrinsic pathways in cervical cancer cells. Several studies have reported that chrysin induces apoptosis through the activation of caspase 3 in different cancer cell lines (Li et al., 2010; Li et al., 2011; Woo et al., 2004). Recent work by Kim et al. (2012) showed that luteolin activates caspase 3 and PARP cleavage, but luteolin-induced cell death can be prevented by a general caspase inhibitor. Another group has demonstrated that luteolin induces caspase-independent cell death in breast cancer through AIF translocation to the nucleus, mediated by activation of ERK and p38 protein kinases (Kim et al., 2012).

Our work shows that quercetin does not activate caspase 3 (Figure 8A and 10), but induces PARP cleavage after 2 hr of incubation (Figure 8B). These results differ from a report by Chien (2009) stating that quercetin-induced apoptosis in breast cancer cells caused by caspase 3 activation and accompanied by reduction of XIAP. PARP cleavage without caspase 3 activation suggests that other proteases are involved in cell death. Vittar, Awruch, Azizuddin, and Rivarola (2010) showed that PARP can be cleaved by calpains. Calpains and cathepsins play a role in caspase-independent cell death (Schrader, Huai, Jockel, Oberle, & Borner, 2010). It also has been reported that cell death mediated by AIF, independent of caspases, is modulated by PARP (Yu et al., 2006). Our results indicate luteolin and apigenin treated cells showed time-dependent increase of cleaved caspase 3 (Figure 9). Apigenin-induced apoptosis involves the activation of both, the intrinsic and extrinsic, apoptotic pathways (Choi & Kim, 2009). However, protein levels of cleaved caspase 3 in flavonoid-treated MDA-MB-231 cells

were weak compared to the positive control (Figure 8A and 9), suggesting that activation of caspase 3 is not the primary mechanism of cell death. White et al. (2012) reported that some natural flavonoids are able to inhibit caspase activity directly, even after staurosporine-induced caspase 3/7 activation. This confirms our hypothesis that flavonoids induce cell death through non-classical apoptotic mechanism.

As mentioned above, some flavonoids do not cause breast cancer cell death (data not shown). Myricetin, daidzein, naringenin, and catechin are not cytotoxic to MDA-MB-231 cells. Our results showed that they also do not induce caspase 3 activation and/or PARP cleavage (Figure 8 and 10). However, naringenin has been reported to induce apoptosis through the activation of caspase 3, 8, and 9 in human leukemia THP-1 cells (Park et al., 2008) and U937 cells (C. Y. Jin et al., 2009). Jin et al. (2010) demonstrated that daidzein induces apoptosis in MCF-7 breast cancer cells through mitochondrial pathway.

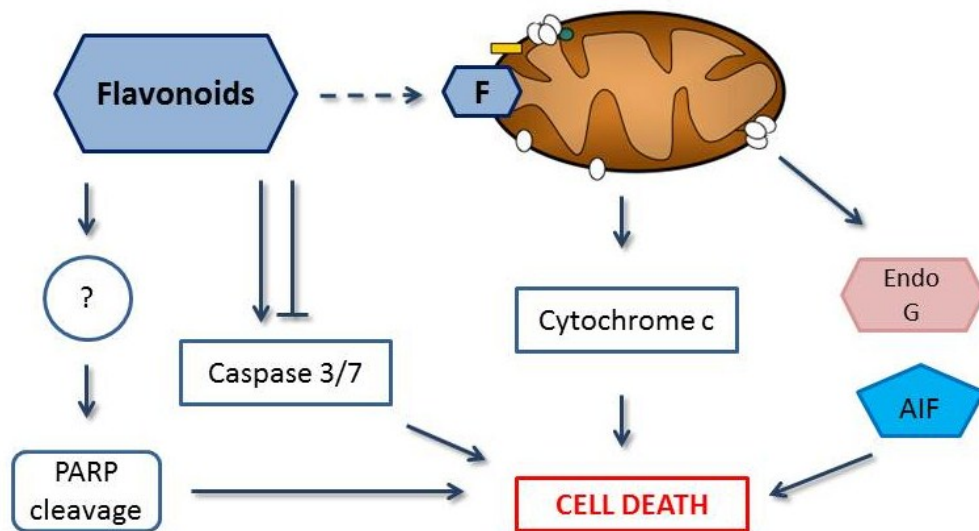
### **Caspase 3/7 and Pro-Apoptotic Bak/Bax are not Essential for Cell Death**

Caspases have been suggested to play an important role in flavonoid-induced cell death (Lee et al., 2008; Liu et al., 2011; Shim et al., 2007). Our results indicated that flavonoids induce equivalent cell death in caspase 3/7 deficient MEFs compared to wild-type (Figure 11), suggesting that caspase-independent cell death is involved in mechanism of flavonoid cytotoxicity.

The pro-apoptotic Bak and Bax proteins belong to Bcl-2 family and are responsible for mitochondria permeabilization during apoptosis (Youle & Strasser, 2008). Cell viability test showed that flavonoids are cytotoxic equally to wild-type and Bak/Bax

deficient BMKs (Figure 12). However, western blot analysis did not reveal activation of caspase 3 and PARP in Bak/Bax double knockout cells, except for PARP cleavage after quercetin treatment (Figure 14). The question arises of why we observe cell death, but not the activation of the main apoptotic proteins in Bax/Bak double knockouts.

Taken together, our results indicated that flavonoids are able to induce cytochrome c release, PARP cleavage, caspase 3 activation, but also inhibit caspase 3/7 activity. A possible explanation of how flavonoids induce cytotoxicity is that these compounds may induce cell death through direct interaction with mitochondria and release of apoptotic proteins, such as AIF and EndoG, which trigger a caspase-independent mechanism of cell death (Figure 15).



*Figure 15.* The summary of possible mechanism of flavonoid-induced cell death.

To better understand the mechanism of caspase-independent cell death, additional studies should be done. Colocalization of AIF and EndoG can be performed in flavonoid-treated MDA-MB-231 and Bak/Bax deficient cells using

immunocytochemistry. We observed PARP cleavage in flavonoid-treated cells despite seeing low levels of caspase activation. It is possible that cathepsins and calpains are responsible for PARP cleavage. However, further studies are needed to prove this. Western blot analysis to activated cathepsins and calpains should be conducted. Also, western blotting can be performed against RIP3 kinase, a marker for necroptosis. Understanding the signaling pathways that control flavonoid-induced cytotoxicity in tumors is critical in order to improve anticancer therapy.

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