

Profiling Flavonoid Cytotoxicity in Human Breast Cancer Cell Lines: Determination of Structure-Function Relationships

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Flavonoids have been shown to be cytotoxic to cancer cells. However, the mechanism of cytotoxicity has not been clearly defined. It has previously been reported that HER2/ERBB2, the estrogen receptor, progesterone receptor, and p53 were required for flavonoid induced cytotoxicity in breast cancer cell lines. We have used a panel of breast cancer cell lines, known to contain as well as be deficient in these signaling pathways, to screen fourteen different flavonoids. Comparing the cytotoxicity for all flavonoids allows us to determine if a structure-functional relationship exists between cytotoxicity and flavonoid, and if a particular signaling pathway is required for cytotoxicity. We show that several flavonoids are cytotoxic to all cell lines including primary mammary epithelial cells tested. The cytotoxic flavonoids are also able to inhibit Mitochondrial Outer Membrane Permeability while at the same time stimulate ATP levels whereas the non-cytotoxic flavonoids are not able to do this. We also show that both cytotoxic and non-cytotoxic flavonoids can transverse the cell membrane to enter MDA-MB-231 cells at different levels. Finally, all flavonoids regardless of their cytotoxicity were able to induce some form of cell cycle arrest. We conclude that for flavonoids to be strongly cytotoxic, they must possess the 2,3-double bond in the C-ring and we believe the cytotoxicity occurs through mitochondrial poisoning in both cancer and normal cells.

Keywords: Apoptosis, Mitochondrial Toxicity, p53, HER2, Cell cycle arrest, Mass spectrometry.

Flavonoids are polyphenolic compounds isolated from a variety of plants, including fruits and vegetables (Figure 1) [1]. They constitute a large portion of compounds found in the plant kingdom with >4000 varieties having been identified to date. These compounds are reported to have a variety of biological activities including anti-oxidant, anti-inflammatory, and anti-tumorigenic properties. Epidemiological studies indicate that a high intake of fruits and vegetables that contain flavonoids may be associated with reduced cancer risk [2]. Flavonoids have also been shown to induce cellular cytotoxicity in cancer cells grown in culture while not killing non-transformed cells[3]. The mechanism of action of these compounds is reported to occur through cell cycle arrest and/or induction of apoptosis. Flavonoids are also able to bind to various signaling proteins and inhibit their actions. These proteins include p53, HER2, EGFR, AKT, ERK1/2. However, to date, the exact molecular mechanism responsible for apoptosis and anti-proliferation/cytotoxicity have not been fully elucidated.

In our study, we have taken advantage of the use of a variety of human breast cancer cell lines to help elucidate the specific mechanism of action required to induce cellular cytotoxicity. A unique advantage for using human breast cancer cell lines is they have been comprehensively analyzed for activity of various signaling pathways [4]. The authors from this study concluded that breast cancer cell lines mimic very closely primary breast tumors and that the signaling pathways have not changed over time in culture. Therefore, we can use the various breast cancer cell lines to mimic the cancer present in an individual to help us determine the mechanism of action of flavonoids.

For our study, we have specifically selected breast cancer cell lines that have been shown to have certain signaling proteins either

present, defective through mutation, or absent (ESR1, HER2/ERBB2, Progesterone Receptor, p53) (Table 1). Some of these same cell lines have been used in previous studies characterizing individual flavonoid action (Table 2) [5-35]. However, a single comprehensive study has not been conducted to date using a variety of flavonoids and breast cancer cell lines.

Table 1: Cell Lines Used in this Study. Signaling components found in individual cell lines are indicated. + means present, - means absent, +/- means weak expression. ESR1-Estrogen Receptor 1, PR-Progesterone Receptor, HER2-Human Epidermal Growth Factor Receptor 2, WT, Wild Type.

Cell Line	BT-474	MCF7	MDA-MB-231	SkBr-3	ZR-75-1
Invasiveness	Strong	Weak	Strong	Strong	Moderate
Gene Cluster	Luminal	Luminal	Basal	Luminal	Luminal
Subtype					
P53	E285K	WT	R280K	R175H	WT
ESR1	+	+	-	-	+/-
PR	+	+	-	-	+
HER2	++	+	-	++	+/-

We used fourteen different flavonoids across five different breast cancer cell lines to help us determine structural-functional relationships for the flavonoids, as well as, to understand if a certain signaling pathway is required for function. For example, some breast cancers are known to be 'triple-negative' and lack ESR1, PR, and HER2 signaling. Therefore, it is important to know if different sub-categories of flavonoids can or cannot kill 'triple-negative' breast cancer cell lines. We have also used Human Mammary Epithelial Cells (HMEC) for comparison to a non-transformed cell type. Flavonoids have previously been reported to have no effect MCF10A cells, a non-transformed cell type [32-34]. MCF10A cells have been immortalized and thus are different than HMEC's, which have a finite life-span in culture. Therefore, we wanted to directly test the effects of flavonoids on a non-transformed cell. We show

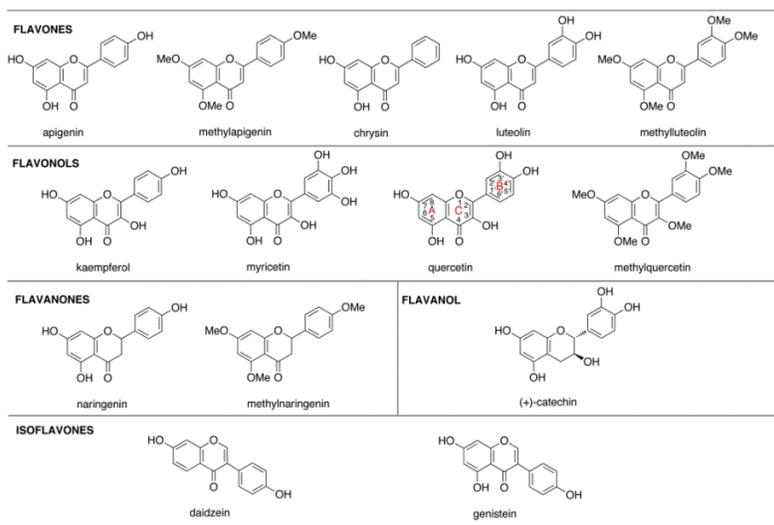


Figure 1: Structures and sub-categories of flavonoids used in this study. Flavonoids are polyhydroxylated flavone (2-phenyl-1,4-benzopyrone) derivatives of plant origin. They are subdivided into six major subcategories (flavanols, flavones, flavanones, flavonols, isoflavonoids, and anthocyanidins). The flavones contain a 2,3-unsaturation in the C ring, and the flavonols possess both the 2,3 unsaturation and a hydroxyl group at the 3 position. The flavanones, by contrast have a saturated 2,3-bond while the flavanols possess a hydroxyl group at the saturated 3 position. The isoflavonoids are isomers of the flavones with the attachment of the B ring at the 3-position rather than the 2-position. The flavonoids may be hydroxylated at positions 3, 5, 7, 3', 4', and/or 5' as shown for the fully hydroxylated myricetin, and the 2,3 double bond may be a single bond, as in naringenin or catechin. Numbering is indicated on quercetin.

Table 2: A survey of literature for effects of flavonoids on breast cancer cell line proliferation and cytotoxicity.

Cell Line	Flavonoid Used	Reference
BT-474	Apigenin	5
MCF-7	Apigenin	6-13,32
	Catechin	
	Genistein	
	Kaempferol	
	Myricetin	
	Naringenin	
	Quercetin	
MDA-MB-231	Apigenin	7, 14-27
	Genistein	
	Kaempferol	
	Naringenin	
	Quercetin	
SK-Br-3	Apigenin	28
ZR-75-1	Apigenin	29-31
	Genistein	
MCF10A/HMEC	Naringenin	32-34
	Quercetin	
	Genistein	

that only certain flavonoids can induce cell death in all cell lines tested, including HMECs, suggesting flavonoids are not dependent on a particular signaling pathway as previously reported.

We measured cell viability in five cancer cell lines and HMECs after treatment with fourteen different flavonoids in two formats. The first format used Promega's Cell Titer Glo (CTG) assay which directly measures ATP levels present in the cell. This assay has previously been shown to indicate a measurement of cytotoxicity/cell death. The CTG assay works based on luminescence principles and we determined that the flavonoids do not interfere with luminescent output (data not shown). We chose this assay over the conventional MTT or MTS assays because the flavonoids absorb light at the same wavelength as the MTT and MTS assays (data not shown) [36,37] which could interfere with proper interpretation of the data. The second assay to measure cellular viability was the trypan blue exclusion (TBE) assay. In this assay, trypan blue is added to cells and counted on a hemocytometer. Live cells do not take up trypan blue whereas dead or dying cells will take up trypan blue due to membrane damage.

We measured cellular viability at 24, 48, and 72 hours after treatment with flavonoids with the CTG Assay (Figure 2 and Supplemental Figure S1). Cellular viability decreased over time

and flavonoid concentration. Maximum cell cytotoxicity was seen after 72 hours of treatment. Statistical data analysis was conducted for CTG assay using a four-factor ANOVA model for drug, cell line, drug concentration, and time. F-tests followed by Tukey's multiple comparison procedure were used to find statistically significant differences in cell death. There was a significant time-dose effect of cytotoxicity of the flavonoids in all cancer cell lines as well as HMECs tested with maximum cell death seen at 72 hours after initial treatment ($p < 0.001$). We were surprised to see that some of the flavonoids appeared to stimulate cell viability at lower concentrations (Figure S1; viability above 100% of DMSO treatment).

Because we were seeing stimulation of cellular viability using the CTG assay, we wanted to know if this was truly an increase in the number of cells or simply an increase in the amount of ATP being produced by the cells since the CTG assay directly measures ATP levels. Therefore we determined cellular viability using the TBE assay and compared the results to CTG assay. We chose to only measure cellular viability at 100 μ M flavonoid and 72 hours of treatment. The results indicate that the two assays do not show the same amount of cellular viability (Figure 3). We have also provided the results in a bar graph comparison format (Supplemental Figure S2). We have used a line to bisect the graph at a 45 degree angle to indicate that if CTG and TBE assays were identical, the data would fall on this line. As can be seen in Figure 4, the majority of the data points lie on the left side of the line indicating a lower TBE.

We calculated IC₅₀ curves for all flavonoids after 72 hour of treatment using the results from the TBE assay. Table 3 shows the calculated IC₅₀s with 95% confidence interval ranges indicated in parenthesis. The IC₅₀ varied significantly across the flavonoids. However, the IC₅₀ was comparable for a single flavonoid across all cell lines tested with the exception of quercetin, methyl-quercetin, and daidzein which showed variability depending on the cell line tested.

Using a combination of the IC₅₀ data and cellular viability data by trypan blue exclusion, we were able to categorize the flavonoids cytotoxic ability (Table 4). Apigenin, m-apigenin, and luteolin are the most effective at inducing cytotoxicity in all cell lines tested.

Table 3: IC50 for flavonoid induced cell death at 72 hours. Values are reported as micromolar. Values in parenthesis are the range for 95% confidence interval. >200 indicate that flavonoids did not induce cellular death.

	BT-474	MCF-7	MDA-MB-231	Sk-Br-3	Zr-75-1
Apigenin	49 (43-56)	2 (0.33-16)	14 (11-19)	32 (23-46)	14 (43-48)
M-apigenin	42 (35-52)	40 (17-94)	33 (27-40)	53 (36-77)	38 (18-62)
Catechin	>200	>200	>200	>200	>200
Chrysin	80 (66-95)	34 (28-40)	40 (35-45)	34 (27-42)	59 (48-72)
Daidzein	154 (129-182)	102 (80-130)	179 (144-222)	97 (69-139)	>200
Genistein	97 (61-154)	74 (52-105)	51 (43-61)	30 (18-53)	81 (68-96)
Kaempferol	77 (62-96)	25 (16-40)	38 (31-47)	48 (40-58)	80 (61-106)
Luteolin	13 (6-27)	5 (14-20)	16 (14-20)	13 (9-20)	32 (28-37)
M-luteolin	>200	>200	21 (12-38)	133 (87-200)	150 (100-225)
Myricetin	>200	>200	>200	>200	160 (77-330)
Naringenin	>200	>200	>200	>200	>200
M-naringenin	69 (58-82)	102 (64-161)	75 (59-96)	72 (54-96)	40 (22-74)
Quercetin	>200	102 (62-161)	101 (82-124)	64 (55-75)	47 (29-77)
M-quercetin	>200	105 (64-171)	54 (39-75)	78 (50-123)	>200

We found that in certain cell lines, quercetin, m-quercetin, m-luteolin, kaempferol and chrysin were also very effective at inducing cytotoxicity, but were moderately effective in other cell lines. Genistein, m-naringenin, and daidzein were very weak at inducing cytotoxicity in all cell lines. Finally, myricetin, naringenin

and catechin were unable to induce cytotoxicity in any cell line tested. These results strongly argue that not all flavonoids are capable of inducing cytotoxicity in breast cancer cell lines whereas those that do induce cytotoxicity had moderate to strong effects.

The compounds apigenin, chrysin, genistein, kaempferol, luteolin, and quercetin, consistently showed a higher cell viability using the CTG assay than the TBE assay for all cell lines tested. This suggested to us that these compounds were capable of stimulating the amount of ATP present in the cells. To confirm this, we treated MDA-MB-231 cells with 25µM of the flavonoids for 72 hours, counted the cells, and seeded the same number of cells for a CTG assay. The flavonoids stimulated the amount of ATP being produced in MDA-MB-231 cells compared to DMSO treated cells (Figure S3). The stimulation varied from 1.5-3.0 fold more ATP per cell.

Table 4: Summary of findings for cell death using TBE assay and IC50 values. Categories were created based upon strong killing (>80% and IC50<50 µM), weak

Kills Strongly (>80%) Non-Selective	Kills Weakly (20-70%) Selective	Does Not Kill (<20%)
Apigenin	Chrysin	Naringenin
M-Apigenin	Genistein	Catechin
Luteolin	Kaempferol	Myricetin
	M-Luteolin	
	Quercetin	
	M-Quercetin	

killing (30-70% and IC50<100 µM), and no killing (<20%)

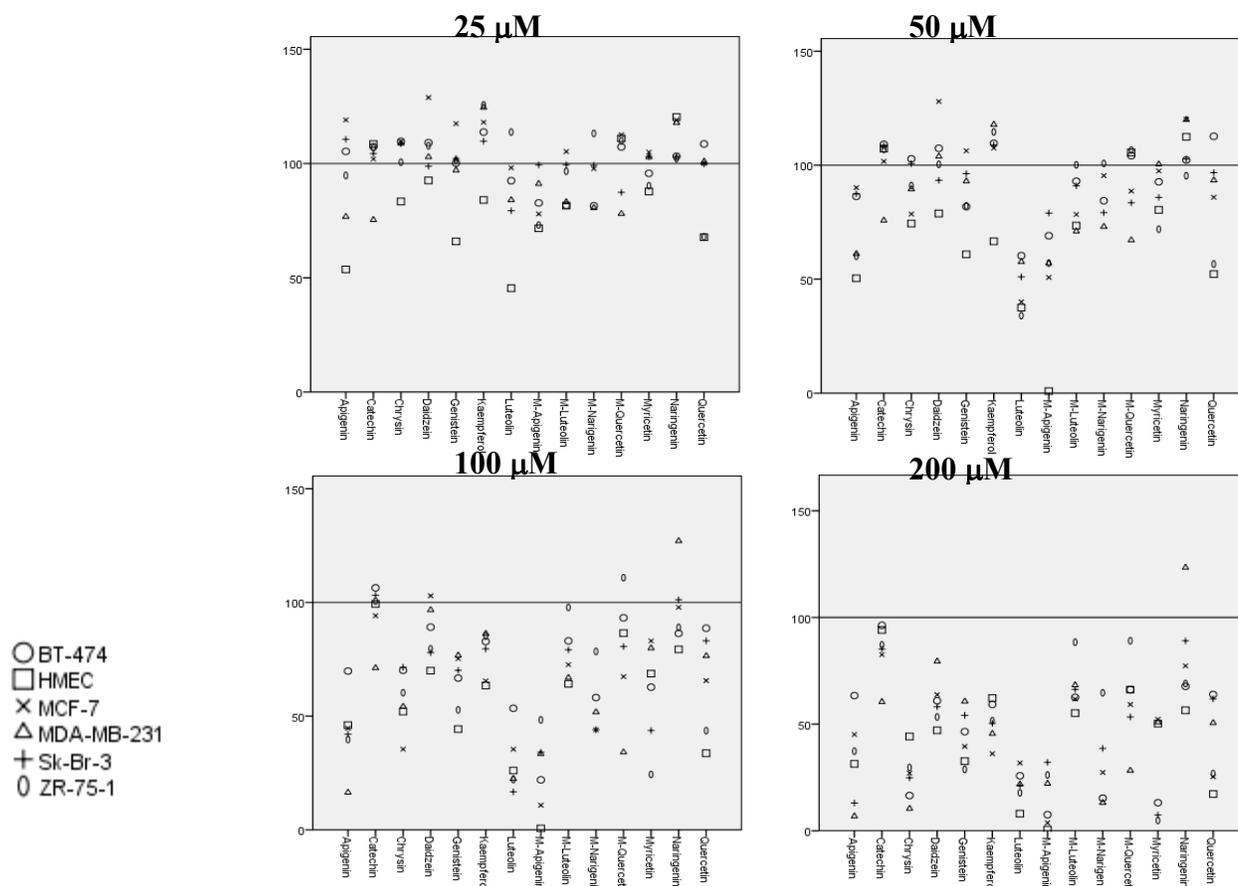


Figure 2: Analysis of cell viability of breast cancer cell lines and primary cells after hour flavonoid treatment. CellTiter-Glo analysis was performed 72 hours after treatment with flavonoids at concentrations indicated. The line in each graph represents the DMSO treated cell group and was set to 100% cell viability. The Y-Axis represents the percent cell viability and the X-Axis represents the flavonoid tested. The key corresponds to the different cell lines used.

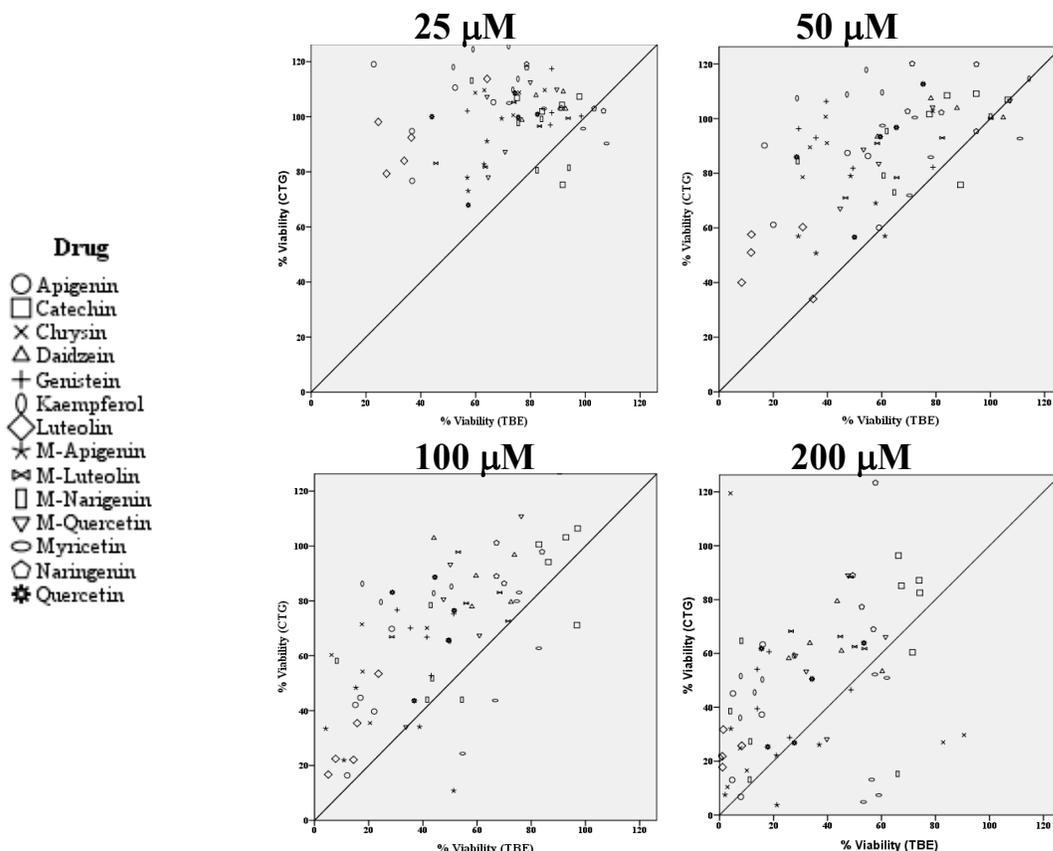


Figure 3: Comparison of cell viability by two methods. Cell Titer Glo (y-axis) and Trypan Blue Exclusion (x-axis) assays were performed as described. Results shown are after 72 hours of flavonoid treatments as indicated for all breast cancer cell lines. A 45 degree line is drawn and symbols shown are above the 45 degree line indicating that results show a higher viability with Cell Titer Glo than Trypan Blue Exclusion.

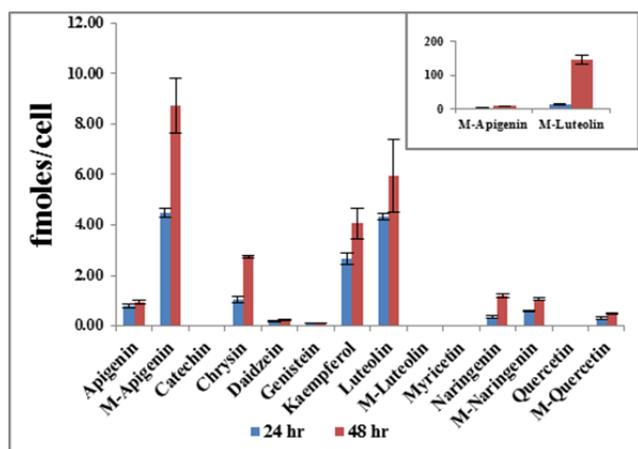


Figure 4: Measuring the amount of flavonoid present in MDA-MB-231 cells. 100 μM flavonoids were added to MDA-MB-231 cells for 24 and 48 hours with the exception of luteolin which was added at 50 μM . Cells were counted and extracted as described and flavonoids were quantified against a standard curve. The inset picture is the quantification of m-luteolin compared to m-apigenin. M-luteolin was 10 to 15-fold higher in cells than any other flavonoid. Results shown are an average with standard deviation from two independent experiments with each experiment having a triplicate run for each flavonoid.

The amount of flavonoid that was taken up by MDA-MB-231 cells was determined (Figure 4). After 24 and 48 hour incubation in 100 μM flavonoid (50 μM for luteolin due to the high amount of cell death), a total cell extract was prepared and LC-MS separation and analysis was performed as described. The results indicate that not all flavonoids were detected in the cell. Most notably, we could not

detect (+)-catechin, myricetin, and quercetin in cell extracts. All other flavonoids were detected in cell extracts. The amount of flavonoids that was detected varied, ranging from 0.5 fmole per cell to 150 fmole per cell. In general, the flavonoids that were able to enter the cell (apigenin, m-apigenin, chrysin, kaempferol, luteolin and m-luteolin) were also the same flavonoids that were able to induce cell death in MDA-MB-231 with similar IC₅₀ values. Flavonoids that could not get into the cell or get in very weakly (catechin, daidzein, genistein, myricetin, and quercetin) did not induce cell death or was very weak at killing cells. The exception to this was naringenin, m-naringenin, and m-quercetin. Naringenin and m-naringenin were able to enter MDA-MB-231 cells at the same levels but naringenin was unable to kill the cells whereas m-naringenin was able to kill the cells. M-quercetin was able to kill MDA-MB-231 cells with very little of the compound entering the cell. M-quercetin was able to enter the cells weakly and induce moderate cell death.

The effect of flavonoids on the cell cycle was assessed by using flow cytometry. All breast cancer cell lines were treated with 100 μM flavonoid and were processed at 24 and 48 hours post-treatment. Table 5 shows the summary of the cell cycle effects seen. All flavonoids had varying abilities to arrest all cell lines at the two time points chosen and were statistically significant ($p < 0.05$) unless indicate (NE). The majority of flavonoids induce cell arrest at G2 with the exception of m-quercetin and m-naringenin which consistently arrested cell lines in G1-phase. It has been previously shown that methylated versions of various flavonoids arrest cells in G1-phase [38] and our data supports these previous findings. We find that cell cycle arrest does not occur with all cell lines for a

particular flavonoid. For example, apigenin and chrysin arrests all breast cancer cell lines in G2-phase with the exception of SkBr3 cells. Another group has reported that apigenin is able to induce cell cycle arrest in Sk-Br-3 cells [29]. We were unable to reproduce this result. However, apigenin does induce cytotoxicity in Sk-Br-3 cells and is similar to what was reported by this group. Therefore, it remains inconclusive in Sk-Br-3 cells whether or not cell cycle arrest occurs upon apigenin treatment.

Kaempferol and quercetin were the only two flavonoids which consistently arrested all cell lines tested at both 24 and 48 hours. Kaempferol is also a very strong inducer of cellular cytotoxicity in all cell lines, whereas quercetin is more selective in its cytotoxicity. Naringenin, daidzein, m-luteolin, are all compounds which are not able to induce cellular cytotoxicity at very high levels. All of these compounds were able to induce cell cycle arrest in some cell lines, but not others. Finally, (+)-Catechin was unable to arrest cells whereas myricetin had mixed cell cycle arrest.

Table 5: Summary of cell cycle arrest identified in all breast cancer cell lines after 24 and 48 hours of 100 μM flavonoid treatment. NE indicates that there was no cell cycle arrest detected that was statistically significant. G2 corresponds to arrest during G2 phase, G1 corresponds to arrest during G1 phase and when indicated was statistically significant (p<0.05). Results shown represent the averages of at least three independent experiments.

Cell Line	BT474		MCF7		MDA-MB-231		SKBR3		ZR-75-1	
	24	48	24	48	24	48	24	48	24	48
Apigenin	G2	G2	G2	G2	G2	G2	NE	NE	G2	G2
M-Apigenin	G2	NE	G2	NE	G2	G2	NE	G1	G2	G2
Catechin	NE	NE	G2	NE	NE	NE	NE	NE	NE	NE
Chrysin	G2	G2	G2	G2	G2	G2	NE	NE	G2	G2
Daidzein	NE	NE	G2	G2	NE	NE	NE	G1	NE	NE
Genistein	G2	G2	G2	NE	G2	G2	G2	G2	G2	G2
Kaempferol	G2	G2	G2	G2	G2	G2	G2	G2	G2	G2
Luteolin	G2	G2	G2	G2	G2	G2	NE	G1	G2	G2
M-Luteolin	G2	G1	NE	G1	NE	NE	G1	G1	NE	G1
Myricetin	NE	NE	NE	G1	G1	NE	G2	G2	G2	G2
Naringenin	G2	G2	G2	NE	G2	G2	NE	NE	NE	NE
M-Naringenin	NE	NE	NE	G1	G1	NE	G1	G1	NE	G1
Quercetin	G2	G2	G2	G2	G2	G2	G2	G2	G2	G2
M-Quercetin	NE	G1	G1	G1	G1	G1	G1	G1	NE	G1

We next compared the ability of flavonoids to induce cell cycle arrest to their ability to induce cytotoxicity at 72 hours. Supplemental Figure S4 shows the comparison of change in G2 compared to cell viability. We determined the 95% confidence interval for G2 and plotted it on the graph as bars. Any data points outside of the 95% confidence interval indicates significant change in G2 DNA content (p<0.05); values to the right of the confidence intervals indicate an increase in DNA content or arrest in G2 whereas values to the left of the confidence intervals indicate a decrease in DNA content or arrest in G1. Taken together, these results suggest that cell cycle arrest may not be a strong indicator of whether or not a flavonoid is able to induce cellular cytotoxicity or that cell arrest precedes cell death.

We measured mitochondria membrane potential ($\Delta\Psi$) after flavonoid treatment using the cationic dye JC-1, a highly specific probe for detecting changes in mitochondrial $\Delta\Psi$. JC-1 forms red aggregates in intact mitochondria. However, permeabilization of the mitochondrial membrane leads to a decrease in the electrochemical gradient across the membrane, resulting in the release of green fluorescent JC-1 monomers into the cytosol. The ratio of red to green fluorescence is calculated from dot plots generated using CellQuest Pro (Figure 5). A 10-fold decrease in the $\Delta\Psi$ was observed in MDA-MB-231 cells treated with apigenin, genistein, kaempferol, luteolin, and quercetin after only 15 minutes of treatment. M-apigenin, catechin, m-luteolin, and m-naringenin had

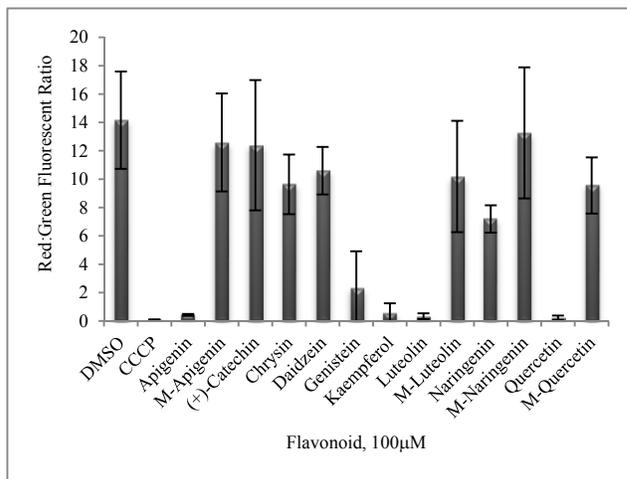


Figure 5: Decrease in mitochondrial membrane potential upon flavonoid treatment. MDA-MB-231 cells were incubated with 100 μM flavonoids or 50 μM CCCP as control and 5μg/mL JC-1 dye for 15 minutes at room temperature in PBS and then vigorously washed with PBS. FACS analysis was performed to evaluate changes in mitochondrial depolarization using fluorescence changes. (A) The ratio of red to green fluorescence was taken and compared to DMSO treated cells and plotted as a bar graph. Error bars represent the standard deviation from five independent experiments. All decreases in the red:green fluorescent ratios showed a statistically significant p<0.05 or better compared to DMSO except m-apigenin, (+)-catechin, m-luteolin, and m-naringenin.

no effect on mitochondrial $\Delta\Psi$, whereas chrysin, daidzein, naringenin, and m-quercetin had a weak but statistically significant (p<0.05) decrease in $\Delta\Psi$.

To determine the mechanism by which cytotoxicity was occurring in breast cancer cell lines, we used Annexin V binding assays to detect loss of phospholipid membrane asymmetry and exposure of phosphatidylserine (PS) at the cell surface in MDA-MB-231 cells. In healthy cells, PS is kept on the inner-leaflet (cytosolic side) of cell membrane. PS appearance in the outer leaflet (extracellular side) is a universal phenomenon associated with cells undergoing apoptosis and is one of the earliest detectable signs of apoptosis [39]. Use of Annexin V coupled to a fluorochrome allows detection of PS in intact live cells if they are undergoing apoptosis. To distinguish intact cells from cells that are necrotic, propidium iodide (PI) is used. In Figure 6, we show the average percentage of cells with standard deviation that are apoptotic or necrotic after treatment with various flavonoids or DMSO as a control (n=3 or greater). We considered apoptotic cells to be the combination of early apoptosis (Annexin V positive only) and late apoptosis (Annexin V and PI positive) as both of these populations increased over time with flavonoid treatment compared to control (Supplemental Figure S3). We did not detect significant PS flipping at 6 or 12 hour time points (data not shown). The increase in apoptotic cells was significant for every treatment tested (p<0.05).

To our knowledge, this is the first comprehensive study of its kind evaluating flavonoid induced cellular cytotoxicity. We used both a variety of breast cancer cell lines and flavonoids to characterize flavonoid induced cellular cytotoxicity. We chose these cell lines based on the presence and absence of a variety of signaling pathways previously reported to be involved in flavonoid induced cellular cytotoxicity (Tables 1 and 2). We chose flavonoids which represent the different sub-groups (Figure 1) in order to determine if there was a structural-functional relationship that existed for cytotoxicity. Our results indicate that apigenin, m-apigenin, and luteolin were able to induce the strongest cytotoxicity in all breast cancer cell lines tested (Table 4). We also show using the

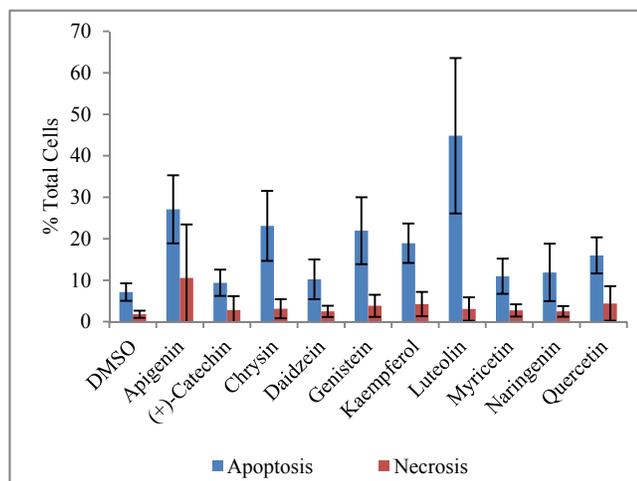


Figure 6: Increase in apoptosis and necrosis by flavonoid treatment. All graphs show averages \pm s.d. from at least three independent experiments. Percent necrotic and apoptotic cells by the Annexin V-Alexafluor 488 assay after 30 h treatment with 100 μ M flavonoid. All increases in apoptosis showed a statistically significant $p < 0.05$ or better compared to DMSO control whereas necrosis was not considered statistically significant.

MDA-MB-231 cells that cytotoxicity is through an apoptotic mechanism due to the strong PS-flipping detected. This is the first characterization of luteolin's and m-apigenin's effects on the breast cancer cell lines. We conclude that since these compounds are able to work equally well on all cell lines and the mechanism of action does not depend on HER2, p53, or the progesterone receptor as has been previously reported as the breast cancer cell lines lacking these proteins (most notably MDA-MB-231 cells) were able to undergo apoptosis as well as cells that have these proteins (MCF7 cells).

The isoflavonoids are considered to be phytoestrogens because of their structural similarity to the mammalian steroid hormone 17 β -estradiol. Both have been reported to bind to the estrogen receptor and activate it [40]. Some breast cancers are dependent on the estrogen-receptor for sustained growth. Therefore, circulating phytoestrogens could be potentially harmful by stimulating growth of breast cancer. Interestingly, consumption of foods rich in phytoestrogens correlates with reduced risk in breast cancer [41]. Our studies indicate that daidzein is a weak inducer of cellular cytotoxicity regardless of cell line tested. However, genistein appears to be selective for ER negative cell lines in the ability to induce cytotoxicity. Genistein was also much stronger at inducing apoptosis as measured by Annexin V staining in MDA-MB-231 cells compared to daidzein.

We have shown that the same flavonoids which induce cytotoxicity in breast cancer cells are able to induce cytotoxicity in HMECs. This is in contrast to previous reports in the literature which have indicated that genistein is unable to decrease cell viability in MCF10A cells (Table 2). However, it should be noted that all studies we have seen in the literature generally have not done a comparison to HMECs or MCF10A cells. We have not tested the MCF10A cell line in any of our studies although we feel that the HMECs are comparable if not better for these studies. Our results suggest that flavonoid action may not directly target cancer cells as previously suggested although this still remains controversial.

Our results show that flavonoids are able to induce cell cycle arrest however the results were inconclusive as to whether cell cycle arrest is important in cell death. For example, methyl-luteolin is able to induce G1 arrest in some cell lines whereas in others it has no

effect. This flavonoid is also very weak in its ability to kill breast cancer cell lines. More importantly, myricetin is able to induce both G1 and G2 cell cycle arrest although it is unable to kill these cells at all. Our strongest killers of breast cancer cell lines (luteolin, apigenin, and m-apigenin) were all able to induce cell cycle arrest in all the cell lines tested. We speculate that perhaps cell cycle arrest could be occurring much earlier than cell death. Indeed, we do not see large amounts of cell death until the 72 hour data point. Therefore, although the results are inconclusive, it is possible that the cells are arresting prior to undergoing cell death. A closer look at the flavonoid induced cell cycle arrest and cell death would help us understand this better.

Our studies indicate that flavonoid induction of cellular cytotoxicity may be occurring through a non-classical apoptotic mechanism. We show here that flavonoids are able to induce PS flipping. However, we have previously reported that flavonoids are able to inhibit caspase-3 and caspase-7 at similar concentrations that induce cytotoxicity in our breast cancer cell lines. We also showed that flavonoids are cytotoxic to cells which lack caspase-3 and caspase-7 [42]. Taken together, these results suggest that a non-caspase form of apoptosis may be occurring although further research is needed.

How do the flavonoids and methylated-flavonoids induce cellular cytotoxicity? Why are some more effective than others? We suggest that flavonoids which are able to induce cytotoxicity may do so in a generalized cellular mechanism. However what is the mechanism for selectivity amongst the flavonoids? We have been able to identify structural features which we believe important in flavonoid induced cytotoxicity. First, in order for a flavonoid to function, it must enter the cell. Our data show that the flavones and some flavanols are able to enter the cell and are cytotoxic, while the flavanones and isoflavones tested were far less effective at killing cancer cells due to their inability to enter the cell. The exception to this is in naringenin and m-naringenin where m-naringenin is able to induce weak cell death.

Our data suggests that the planarity of the benzopyran nucleus may be a key structural element in determining the ability of the flavonoids to cross the cell membrane and induce cytotoxicity. For example, we note that both naringenin and (+)-catechin are identical to apigenin and quercetin, respectively, with the exception of the 2,3-double bond on the C-ring. However, both naringenin and (+)-catechin do not induce cytotoxicity in cell lines tested nor do they enter the cell. Isoflavones possess the 2, 3-double bond but are also weaker in their ability to induce cytotoxicity, presumably due to the location of the B-ring's position off of carbon 3 instead of carbon 2. Taken together, these observations suggest that flavonoid cytotoxicity requires the compounds to be planar in nature, possess the 2,3-double bond on the C-ring and the B-ring to be attached at the 2-position of the benzopyran core.

We also see cellular cytotoxicity occurring with the methylated version of some of the flavonoids. Of the methylated flavonoids, methyl-apigenin was able to induce cytotoxicity at the equivalent level of apigenin, whereas methyl-luteolin and methyl-quercetin were effective in some cell lines and not others. Previous studies have shown that the methylated versions of various flavonoids tend to be more potent than their unmethylated counterparts [43]. Given the comparison data that we have generated here, further investigation is warranted as it would appear that not all methylated compounds can induce cell death. Whether or not this is due to their ability to cross the cell membrane of some cells and not others remains to be tested. We have not determined the concentration of flavonoids in other cell lines tested but it would be interesting to

look at BT-474 and Zr-75-1 cell lines as these cells were the least affected by the m-luteolin and m-quercetin.

We show that ATP levels are higher in cells after flavonoid treatment (Figure 3). We attribute this to stimulation of ATP production. However, it has also been recently shown that a variety of flavonoids (luteolin, quercetin, kaempferol, and apigenin) can block fatty acid synthase [44]. These same flavonoids are also the same ones that we show increase ATP levels in cells. Whereas, the flavonoids that were weakest in the inhibition of fatty acid synthase (naringenin, catechin, myricetin, and genistein) are also some of our weakest at increased ATP levels.

In contrast to the ATP production, we also show that these same flavonoids are also able to rapidly decouple mitochondrial membrane potential. This should prevent the mitochondria from producing ATP. Taken together with the inhibition of fatty acid synthase, we would not expect to see the large increase in ATP production that we are seeing. Therefore, the increased ATP output seen after flavonoid treatment could be coming from increased glycolysis although we have not directly shown this. How would flavonoids stimulate glycolysis? AMP Kinase (AMPK) serves as the cellular energy sensor by monitoring ATP levels necessary for cellular growth and proliferation. AMPK is a cellular energy sensor which monitors that cells have enough ATP present for cellular growth and proliferation [45]. If ATP levels drop, AMPK is activated through binding of AMP to the gamma subunit of the enzyme to stimulate phosphofructokinase and subsequently glycolysis. A recent report has shown that quercetin is able to stimulate AMP Kinase (AMPK) [46]. We speculate that the other flavonoids may also be able to stimulate AMPK although this remains to be tested.

Medicinal plants have played pivotal roles in the development of new drugs to treat human diseases. Some of the earliest forms of chemotherapy originate from natural products derived from both plants and marine organisms. It is no surprise to see a resurgence in the investigation of natural products for their anti-cancer use. Flavonoids have been shown to be potent bioactive molecules that possess anticarcinogenic effects. Flavonoids have also emerged as potential chemopreventative candidates for cancer. Despite this promise, contradictory results regarding molecular mechanisms of action have been reported from many laboratories. Although results from *in vitro* experiments are not always predictive of medicinal utility, they constitute a valuable tool for studying the effects of the drug candidate on molecular targets involved in tumor growth and survival. More studies are clearly needed to resolve the conflicting data, to more fully understand the mechanism(s) of anti-cancer activity of flavonoids, and to evaluate their potential as therapeutic agents. As proteins that interact with flavonoids are identified, these discoveries will provide the basis for a starting point for rational drug design.

Experimental

Materials: Flavonoids and iso-flavonoids were purchased from Indofine or Alexis Biochemicals and stocks were prepared at 50mM in dimethyl sulphoxide (DMSO) with the exception of methyl-luteolin which was prepared at 25mM in DMSO. The compounds were used at 25 μ M, 50 μ M, 100 μ M, and 200 μ M for all experiments. The maximum DMSO concentration used in experiments was 0.4% (200 μ M flavonoid treatment). DMSO at this level did not have an effect in any assays conducted compared to a no DMSO treatment (data not shown). Propidium Iodide was purchased from Sigma. RNase was purchased from Fisher. Trypan Blue was purchased from Hyclone.

Cell Culture: The following human breast cancer cell lines were purchased from ATCC: BT-474, MCF7, MDA-MB-231, SK-BR-3, and ZR-75-1. BT-474, SK-BR-3, and ZR-75-1 were routinely maintained in RPMI 1640 with L-glutamine (Hyclone), supplemented with 10% Bovine Growth Serum (BGS) and 1X antibiotic/antimycotic (Hyclone). MCF7 and MDA-MB-231 cells were maintained in DMEM with high-glucose, L-glutamine, and sodium pyruvate (Hyclone), supplemented with 10% Bovine Growth Serum (BGS) and 1X antibiotic/antimycotic (Hyclone). All cell lines were grown at 37°C in a humidified atmosphere containing 5% CO₂ and passaged based on recommended dilutions and confluencies from ATCC. Human Mammary Epithelial Cells (HMEC) were purchased from Lonza and were grown in recommended media with supplements from Lonza.

Cell viability assays: Cell viability was determined using the cell titer glo assay (CTG, Promega) or trypan blue exclusion assay (TBE). For the CTG assay, 0.5×10^4 cells/well were seeded in 96-well white plates (BD Falcon). 24 hours later, media was changed and cells were exposed to various concentrations of flavonoids. Cell titer glo assays were initiated at 24-, 48- and 72-h after exposure to compounds following the manufacturer recommend protocol. Cells were incubated at room temperature on a variable speed shaker for 10 minutes with the CTG reagent, mixed briefly by pipetting, and extracts were centrifuged at 300xg to remove bubbles. Plates were read in a Veritas 96 well luminometer (Turner Biosystems) or a Glo-Max II Multimode Plate Reader (Promega).

For the TBE assay, 0.5×10^5 cells/well were seeded in 6-well culture dishes. 24 hours later, media was changed and cells were treated with 100 μ M flavonoid or DMSO as a control. Cells were harvested by trypsinization at 24, 48, and 72 hours post treatment and resuspended in 1 mL of media. Cells were briefly vortexed and an equal volume of cell suspension was combined with an equal volume of 0.4% trypan blue in PBS. Live cells (lacking trypan blue) were counted using a hemocytometer.

Quantification of Flavonoids in MDA-MB-231 Cells: To measure the amount of flavonoids in MDA-MB-231 cells, 3×10^6 cells were plated in a 10cm dish and 24 hours later treated with 100 μ M flavonoids or DMSO as a control. For luteolin, we used 50 μ M because 100 μ M killed too many cells making the quantification impossible to do. After 24 and 48 hours of treatment, cells were trypsinized and collected in media and centrifuged at 100 x g. Media was removed and cells were resuspended in PBS. Cell counts were taken in the presence of Trypan Blue using the BioRad TC 10 cell counter. Cells were then pelleted a second time at 100 x g and PBS was removed. Cells were resuspended in 5 mLs of ice cold 40% Methanol, vortexed, placed on ice for 15 minutes and vortexed a second time. Cell debris was pelleted at 3,000 x g for 10 minutes and supernatant was transferred to a new vial for LC-MS analysis. The pellet was resuspended in another 1 mL of 40% methanol, vortexed, placed on ice for 15 minutes and vortexed a second time. The debris was pelleted and the supernatant was transferred to a vial for LC-MS analysis. We prepared standard curves using purified flavonoid diluted to final concentrations of 75 μ M, 50 μ M, 10 μ M, 1 μ M, and 0.1 μ M in 40 % MeOH. An Agilent 1200 Series HPLC was coupled to an Agilent QTOF6520 was used. 1 μ L of sample was separated on a 150mm x 2.1 mm Agilent Cogent Bidentate C18 column. A binary mobile phase system of solvent A (0.1% formic acid) and solvent B (acetonitrile) had a flow rate of 0.5 mL/min. The gradient began with 30% B for 2 min and increased linearly to 95% B at 10 min, at which time the gradient decreased linearly to 30% B at 15 min to return to the initial starting conditions. MS data was collected in full scan positive mode over the mass range of

100–1000 *m/z*. Ion voltages and gas settings were as follows: fragmentor, 150 V; skimmer, 65V; drying gas, 8L/min; gas temperature, 350 C. Agilent MassHunter Acquisition software version B.0.4 was used for data acquisition and analysis. Flavonoids were quantified using Agilent MassHunter Quantitative Analysis software from a standard curve generated. Results are reported as pmole of flavonoid per cell based upon cell counts. Results are from two independent experiments that were run in triplicate.

Apoptosis Assay: Apoptosis in MDA-MB-231 cells was assayed by annexin V and propidium iodide (PI) co-staining using an Annexin-V-AlexaFluor 488 staining kit (Invitrogen) following a standard protocol [47]. 1×10^6 MDA-MB-231 cells were plated in a 10cm dish and 24 hours later 100 μ M of different flavonoids were added. Cells were analyzed 30 and 48 hours after flavonoid treatment. Cells were harvested by addition of 0.25% trypsin and 5.3 mM EDTA for 2 minutes at 37°C. Trypsin and EDTA were inactivated by addition of complete medium. Cells were collected by centrifugation at 100 x g and resuspended in 1 mL of room temperature annexin-binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl₂, pH 7.4). 100 μ L of the cell suspension was transferred to 1.5 mL eppendorf tube containing 10 μ L of AlexaFluor 488-conjugated annexin V. The cells were incubated at room temperature for 15 minutes and then 400 μ L of annexin-binding buffer plus 10 μ L of PI (50 μ g/mL) was added to the cells. The stained cells were then analyzed by flow cytometry using a BD FACSCaliber instrument. The instrument was set for FL 1 (annexin V) vs FL3 (PI) bivariate analysis. Data from 10,000 cells/sample was collected and dot plots of FL1 vs FL3 were generated. The quadrants were set based on the population of healthy, unstained cells in untreated samples compared to cells treated with 1 μ M staurosporine for 6 or 24 hours. CellQuest Pro was used to calculate the percentage of the cells in the respective quadrants. A minimum of three different experiments was performed.

Analysis of mitochondrial transmembrane potential: Loss of mitochondrial transmembrane potential was monitored by flow cytometry using the JC-1 assay using manufacturer's recommended protocol (Invitrogen). Briefly, 1×10^6 MDA-MB-231 cells were treated with 100 μ M flavonoids for 15 minutes or 50 μ M CCCP (carbonyl cyanide 3-chlorophenylhydrazone) as a positive control treatment. Cells were pelleted, washed with PBS, and resuspended in 500 μ L of PBS. Mitochondrial transmembrane potential was then measured using a FACSCaliber instrument (BD Biosciences) equipped with CellQuest and 10,000 events were collected for analysis. The ratio of red to green fluorescence was calculated.

Cell cycle analysis: Cell cycle analysis was conducted following a previous published protocol [48]. Briefly, 0.5×10^5 MDA-MB-231 cells were seeded in 6-well culture dishes. 24 hours later, cells were treated with 100 μ M of various flavonoids. 24 and 48 hours after treatment, cells were harvested via trypsinization, washed with cold

PBS and processed for cell cycle analysis. Briefly, the cells were fixed in absolute ethanol and stored at -20°C for later analysis. The fixed cells were centrifuged at 1000 rpm and washed with cold PBS twice. RNase A (20 μ g/mL final concentration) and propidium iodide staining solution (50 μ g/mL final concentration) was added to the cells and incubated for 30 min at 37°C in the dark. The cells were analyzed using a FACSCaliber instrument (BD Biosciences) equipped with CellQuest and 10,000 events collected for analysis.

Statistical analyses: Statistical data analysis was conducted for the CTG assay using a four-factor ANOVA model for drug, cell-line, drug concentration and time. F-tests followed by Tukey's multiple comparison adjustment were utilized to identify statistically significant differences in cell death. To compare cell death as measured by CTG and TBE, p-values were calculated from two-sided Student's t-tests followed by Hochberg multiple comparison adjustments. Only adjusted p-values of $p < 0.05$ were considered statistically significant.

For comparison of cell viability to cell cycle arrest, 95% confidence intervals were computed for G2 fold change of one (no change) and plotted against cell viability as determined by TBE assays.

Dose-response data were analyzed using sigmoidal curve fits in Prism (GraphPadSoftware, Inc) with variable slope to determine IC50 values. The top part of the curve was set to 100% response (0% viability) and the bottom part of the curve to 0% (100% viability). IC50 values are reported with 95% confidence intervals.

For comparison between two groups in all other assays, the data were analyzed using the two-sided, two independent sample Student t-test with 95% confidence intervals reported. A p-value of $p < 0.05$ was regarded as statistically significant.

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Supplementary data - Figure S1 is the full figure for data presented in Figure 2. Figure S2 is the bar graph representation of data presented in Figure 3. Figure S3 is the quantification of ATP levels in normalized cells. Figure S4 is a comparison of cell cycle to cell viability. Table S1 is a summary of statistical significant differences between CTG and TBE assays after Hochberg analysis applied. Figure S5 is the representative dot plots of Annexin V and PI Staining of MDA-MB-231 cells treated with various flavonoids or DMSO as control for 30 and 48 hours.

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