Published in final edited form as:

Arch Biochem Biophys. 2014 December 1; 563: 108–117. doi:10.1016/j.abb.2014.06.034.

Involvement of ER stress and activation of apoptotic pathways in fisetin induced cytotoxicity in human melanoma

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Abstract

The prognosis of malignant melanoma remains poor in spite of recent advances in therapeutic strategies for the deadly disease. Fisetin, a dietary flavonoid is currently being investigated for its growth inhibitory properties in various cancer models. We previously showed that fisetin inhibited melanoma growth *in vitro* and *in vivo*. Here, we evaluated the molecular basis of fisetin induced cytotoxicity in metastatic human melanoma cells. Fisetin treatment induced endoplasmic reticulum (ER) stress in highly aggressive A375 and 451Lu human melanoma cells, as revealed by up-regulation of ER stress markers including IRE1 α , XBP1s, ATF4 and GRP78. Time course analysis indicated that the ER stress was associated with activation of the extrinsic and intrinsic apoptotic pathways. Fisetin treated 2-D melanoma cultures displayed autophagic response concomitant with induction of apoptosis. Prolonged treatment (16 days) with fisetin in a 3-D reconstituted melanoma model resulted in inhibition of melanoma progression with significant apoptosis, as evidenced by increased staining of cleaved Caspase-3 in the treated constructs. However, no difference in the expression of autophagic marker LC-3 was noted between treated and control groups. Fisetin treatment to 2-D melanoma cultures resulted in phosphorylation and activation of the multifunctional AMPK-activated protein kinase (AMPK) involved in the regulation of diverse cellular processes, including autophagy and apoptosis. Silencing of AMPK failed to prevent cell death indicating that fisetin induced cytotoxicity is mediated through both AMPK-dependent and -independent mechanisms. Taken together, our studies confirm apoptosis as the primary mechanism through which fisetin inhibits melanoma cell growth and that activation of both extrinsic and intrinsic pathways contributes to fisetin induced cytotoxicity.

Keywords

Fisetin; cytotoxicity; melanoma

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Introduction

Despite recent advances in the treatment of melanoma, the success of systemic therapy for metastatic melanoma is limited (1, 2). Melanoma cells evade elimination due to their complex phenotype, an efficient DNA repair system, protection from endoplasmic reticulum (ER) stress, and presence of pro-survival machinery that maintains cancer cells viability by counteracting apoptotic stimuli during tumor development and metastasis (2-4). The two major apoptotic pathways recognized as the death receptor (extrinsic) and mitochondrial (intrinsic) pathways play crucial roles in tumor progression as well as resistance to therapeutic strategies. Representatives of the Bcl-2 family of proteins and inhibitors of apoptosis are widely overexpressed or overactivated in melanoma, resulting in complex blockades of the apoptotic pathways at the level of cell death initiation and execution (5). Moreover, adaptation to ER stress has been proposed to be a driver of malignancy and resistance to therapy in human melanoma (6). In higher eukaryotes, the ER-stress response is mediated by at least three main stress sensors all located at the ER namely the Inositol-requiring enzyme 1 α (IRE1 α), the PKR-like ER kinase (PERK), and the activating transcription factor 6 (ATF6) (7). Active IRE1 leads to the splicing of transcription factor XBP1s, resulting in the removal of 26 nucleotides and translation of an active form (7). PERK activation induces the translation of the transcription factor ATF4, dampening the translation initiator factor eIF2 α activity, leading to decreased translation. The transcriptionally active form of ATF6 is produced by proteolysis (7). Activation of these pathways triggers the ER-stress response characterized by the transcription of genes that increase the folding capacity of the ER and decrease the synthesis of proteins involved in ER overload. If these mechanisms of adaptation are insufficient to recover ER function, both the intrinsic and extrinsic pathways for apoptosis can become activated, and damaged cells undergo cell death (8). The role of autophagic cell death also referred to as programmed cell death type II is still under debate in the context of its cancer protective effects (9). Accumulating evidence suggests that autophagy serves a largely cytoprotective role in physiologically relevant conditions. The cytoprotective function of autophagy is mediated in many circumstances by negative modulation of apoptosis which in turn serves to inhibit autophagy (10). It is now being recognized that autophagy is not only a survival response to growth factor or nutrient deprivation, but also an important mechanism for tumor cell suicide (11). Inhibition of cytoprotective autophagy by genetic or pharmacological means has been shown to enhance anticancer drug-induced cell death. In contrast, disruption of autophagy, which reduces cellular fitness, can actually promote tumorigenesis (12). Furthermore, induction of autophagy has been implicated as a cancer protective mechanism in flavonoid induced cell death in various cancer cell lines (13). Thus, modulating the basal levels of autophagy resulting in enhanced cell death may be a useful approach in cancer therapeutics.

Low cellular energy levels with high AMP levels activate 5' AMP-activated protein kinase (AMPK), a heterotrimeric protein kinase composed of an catalytic and and regulatory subunits. AMPK plays a critical role in tumorigenesis through regulation of cell proliferation and apoptosis by phosphorylating key substrates, such as p53 and p27Kip1 (14). In addition, AMPK is vital to the induction of autophagy through mTOR-dependent and -independent

mechanisms (15). AMPK activation was shown to be involved in vincristine induced apoptosis in melanoma cells (16). Induction of AMPK activity inhibits the ability of melanoma cells to form colonies in an anchorage-independent manner (16).

The steady increase in the incidence of melanoma over the last several years combined with the inherent difficulty to treat the disease once it has metastasized calls for novel preventative and therapeutic regimens. Epidemiological evidence suggests that a plant-based diet rich in flavonoids is effective against cancer (17). Fisetin (3,7,3',4'-tetrahydroxyflavone), a dietary flavonoid present in several fruits and vegetables is being investigated for its antiproliferative activity against several human cancers (18, 19). Recent data from our laboratory and others demonstrated that fisetin can inhibit tumor growth both in *in vitro* and *in vivo* models (20). Here, we examined the effect of fisetin on cellular energy balance and AMPK signaling and delineated the mechanism(s) through which fisetin induces melanoma cell death. We show that fisetin treatment to melanoma cells resulted in apoptotic cell death mediated through activation of the extrinsic and intrinsic pathways. Furthermore, the autophagic response that accompanies apoptosis in fisetin treated melanoma cells is transient and appears to be independent of AMPK signaling.

MATERIALS AND METHODS

Materials

Fisetin and 3MA were purchased from Sigma Chemical Co. (St. Louis, MO). All antibodies except ATF4 were obtained from Cell Signaling Technology (Danvers, MA). ATF4 was purchased from Santa Cruz Biotechnology (Dallas, TX).

Cell culture/treatment

A375 (ATCC, VA) and 451Lu human melanoma cell lines kindly provided by Dr. Meenhard Herlyn (Wistar Institute, PA) were cultured in DMEM and MEM from Gibco (Carlsbad, CA), with 10% FBS and 1% penicillin-streptomycin, at 37°C with 5% CO₂ in a humid environment. For dose/time-dependent studies, cells (70% confluent) were treated with fisetin dissolved in DMSO (0-80 µM) for specified time points at 37°C in media and harvested for further studies.

Apoptosis assay/flowcytometry

451Lu cells treated with/without fisetin for 48 h were processed, as per manufacturer's instructions for labeling with fluorescein-tagged dUTP nucleotide and propidium iodide using the APO-DIRECT™ kit (Phoenix Flow Systems, CA) and analyzed using the ModifitLT V3.0 software.

Apoptosis assay/Annexin staining

The annexin-V-Fluos staining kit was used for the detection of apoptotic and necrotic cells according to vendor's protocol. This kit uses a dual-staining protocol in which the apoptotic cells are stained with annexin-V (green fluorescence), and the necrotic cells are stained with propidium iodide (PI) (red fluorescence). Cells were grown to ~70% confluency and treated

with fisetin (40 μ M: 24h). The fluorescence was detected by Nikon Eclipse Ti fluorescent microscope. Images were captured with an attached camera.

Enzyme Linked Immunosorbent Assay

451Lu cells treated with/without fisetin for 48 h were evaluated for caspase activity using Human Caspase-8 and Human Caspase-9 Elisa kits purchased from Bender MedSystems (San Diego, CA) as per manufacturer's protocol. The ATP/cAMP levels were determined by Cyclic AMP XP™ Assay kit (Cell Signaling Technology), where the magnitude of absorbance was inversely proportional to the quantity of sample cAMP.

Preparation of cell lysate

After treatment of cells with fisetin, whole, cytosolic and nuclear lysates were prepared and western blot analysis was performed as described previously (20).

Measurement of ROS generation

The OxiSelect™ Intracellular ROS Assay Kit obtained from Cell Biolabs Inc. (San Diego, CA) provides a cell-based assay for measuring primarily hydrogen peroxide, along with hydroxyl, peroxy and other ROS levels within a cell. The assay employs the fluorogenic probe DCFH-DA, which diffuses into cells and is deacetylated by cellular esterases into the non-fluorescent DCFH. In the presence of ROS, DCFH is rapidly oxidized to highly fluorescent DCF. Cells pre-incubated with DCFH-DA at 37 °C for 45 min were treated with/without fisetin (60 μ M) for specified times. Fluorescence was evaluated on a Synergy H1 (BioTek) multi-mode microplate reader at 480/530nm (excitation/emission) using Gen5 2.0 software (BioTek).

Measurement of Nitric Oxide (NO) generation

NO levels were measured as per manufacturer's protocol employing the OxiSelect™ Intracellular Nitric Oxide Assay Kit obtained from Cell Biolabs Inc. (San Diego, CA). Briefly, cells treated/untreated with fisetin were incubated with the cell-permeant NO probe which passively diffuses into cells and is deacetylated by cellular esterases to a non-fluorescent intermediate. Cells were treated with/without fisetin after 45 min incubation with the probe. When intracellular NO encounters the non-fluorescent intermediate, it rapidly oxidizes to a highly fluorescent, triazolo-fluorescein analog. The fluorescence was evaluated on a Synergy H1 (BioTek) multi-mode microplate reader at 480/530nm (excitation/emission) using Gen5 2.0 software (BioTek).

Immunocytochemistry

Immunocytochemical analysis of 451Lu cells, seeded in 2 chamber tissue culture slides treated with/without fisetin was done using FITC-LC-3 antibody as described elsewhere. Briefly, 451Lu cells treated with fisetin (60 μ M: 24h) were fixed in 1% paraformaldehyde. After incubation with 3% H₂O₂ in methanol for 20 min, and blocking with Sniper solution (Biocare Medica, Concord, CA), cells were incubated o/n with anti-LC-3 antibody after which appropriate fluorophore conjugated secondary antibody was added for 1 h. Slides were mounted with AntiFade/Dapi mounting medium. Staining was visualized using the

Nikon Eclipse Ti microscope. For immunohistochemical analysis, formalin fixed sections from melanoma constructs were blocked (2% goat serum/1XPBS) for 30 minutes after deparaffinization and rehydration in xylene and a graded series of ethanol. After incubation with specific primary antibody overnight and HRP-conjugated secondary antibody for 2 h, immunoreactive complexes were detected using 3,3'-diaminobenzidine (Dako Corp., Carpinteria, CA). Sections were counterstained with haematoxylin and visualized on Nikon Eclipse Ti microscope. Images were captured by a camera attached to computer. Figures were composed using ADOBE PHOTOSHOP 7.0 (Adobe Systems, CA).

RNA isolation and qPCR

Total RNA was extracted from cells using RNeasy kit (Qiagen, Germantown, MD), and reverse transcribed with iScript Reverse transcription supermix kit (Biorad, Hercules, CA). cDNA (1-100ng) was amplified in triplicate using gene specific primers. Threshold cycle (C_T) values obtained from the instrument's software were used to calculate the fold change of the respective mRNAs. C_T was calculated by subtracting the C_T value of the housekeeping gene (18s) from that of the mRNA of interest. C_T for each mRNA was then calculated by subtracting the C_T value of the control from the experimental value. Fold change was calculated by the formula 2^{-C_T} .

Melanoma Skin Model

The full thickness melanoma skin model (MLNM-FT-A375) obtained from MatTek Corporation (Ashland, MA) consists of human malignant melanoma cells (A375), normal, human-derived epidermal keratinocytes and fibroblasts cultured to form a multilayered, highly differentiated epidermis with A375 melanoma cells at various stages of melanoma malignancy. At different stages of the culture, the tissue exhibits radial growth phase, vertical growth phase, or metastatic melanoma phenotype. The melanoma skin tissue was supplied as a single well tissue culture plate insert with each insert containing functionally and metabolically active reconstituted skin along with melanoma cells, shipped at 4°C on medium-supplemented, agarose gel. Upon receipt, the melanoma skin tissue was equilibrated at 37°C, 5% CO₂, for 24 h and maintained in MMT media. Throughout the experiment, the tissue was maintained in 6-well culture plates at the air-liquid interface with the lower dermal side of the tissue exposed to media and the upper epidermal stratum corneum exposed to air. Fresh media was supplemented every alternate day with/without fisetin (80 μM) after which samples skins were fixed in 10% formalin or OCT at 0, 4, 8, 12 and 16 days for further studies.

ER stress response Assay

To determine the ER stress response induced by fisetin, we utilized the SelectScreen® Cell-based Pathway Profiling Service provided by Life Technologies (Madison, WI). HeLa cells are engineered to express β-lactamase (*bla*) under the control of ER stress response element (ESRE-*bla* cells) through the ATF6 receptor. Cells are loaded with an engineered fluorescent substrate containing two fluorophores, coumarin and fluorescein. In the absence of *bla* expression, the substrate molecule remains intact and excitation of coumarin results in fluorescence resonance energy transfer to the fluorescein moiety and emission of green light.

However, when *bla* is expressed, the substrate is cleaved, separating the fluorophores, and resulting in a blue fluorescent signal. The response ratio is then calculated and plotted. Briefly, cells were assayed in a 384-well format at 5,000 cells/well. Following overnight incubation, serial dilutions of fisetin were applied to the wells (0.1 % final DMSO) for 30 minutes prior to the treatment with tunicamycin (positive control) for 5 h and loading the wells with LiveBLAzer™- FRET B/G Substrate for 2.5 h. Emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader. Response Ratios were calculated by dividing the 460/530 ratios of the treated wells from the 460/530 ratios obtained with the untreated control wells after which the graph was plotted.

cAMP Assay

cAMP concentrations were measured by using cyclic AMP XP™ assay kit (Cell Signaling Technology). In this assay, cAMP found in test sample competes with a fixed amount of HRP-linked cAMP for binding to an anti-cAMP XP® Rabbit mAb immobilized onto a 96-well plate. Briefly, 50 µl of fisetin treated 451Lu cell lysates were transferred into microtiter plates, and cAMP concentrations were measured according to the protocol provided by the kit. Following incubation and washing to remove excess sample cAMP and HRP-linked cAMP, HRP substrate TMB was added to develop color. Because of the competitive nature of this assay, the magnitude of the absorbance for this developed color is inversely proportional to the quantity of sample cAMP and is a direct measure of cellular ATP levels.

RNA interference studies

Transfection of A375 cells was performed by electroporation using an Amaxa Nucleofector according to the manufacturer's protocol (Amaxa Biosystems, Germany) with siRNAs against AMPK and scrambled siRNA (SantaCruz, CA). Transfected cells were harvested 24 h after fisetin treatment for further studies.

Statistical analysis

Results were analyzed using a two-tailed Student's unpaired *t* test, using GraphPad QuickCals software and *p*<0.05 was considered statistically significant.

Results

Fisetin induces apoptosis in melanoma cells

We first evaluated the mode of cell death in fisetin treated melanoma cells. Upon initiation of apoptosis, phosphatidylserine loses its asymmetric distribution in the phospholipid bilayer and translocates to the extracellular membrane, where it can be detected with fluorescently labeled Annexin V. A375 cells treated with fisetin (60 µM: 24 h) showed increased fluorescent staining as compared to the untreated controls indicating that fisetin induces apoptotic cell death in melanoma cells (Fig.1A). Flowcytometric analysis of fisetin treated A375 and 451Lu melanoma cells demonstrated a dose-dependent increase in the number of cells undergoing apoptosis (Fig.1B). The efficacy of fisetin in the induction of apoptosis varied with cell type as A375 cells were more susceptible to fisetin treatment compared to 451Lu cells (14.7% versus 5.54%; 60µM:48 h) (Fig.1B).

Fisetin induces apoptosis through activation of the extrinsic and intrinsic apoptotic pathways in melanoma cells

Fisetin induced the expression of death receptors TNFR-1 and TNFR-2 (Fig.2A) while no significant effect was observed on DR-3, 4 and 5 (data not shown). The expression of proapoptotic Bax was upregulated while the anti-apoptotic Bcl-2 protein expression was substantially decreased with fisetin treatment in both cell lines (Fig.2B), indicating that there is concomitant activation of both extrinsic and intrinsic pathways of apoptosis (SFig.1). To verify these findings, we performed ELISA and assessed the activity of Caspases -8 and -9 in fisetin treated 451Lu cells. As expected, increased Caspase-8 and -9 activities were evident with all doses of fisetin (Fig.2C&D). Intense staining for Caspase-3 accompanied with PARP cleavage established the mechanistic basis of apoptosis observed in fisetin treated melanoma cells (Fig.2E&F).

Fisetin inhibits ROS and augments NO generation in melanoma cells

Since flavonoids are known to induce apoptosis through generation of ROS, we investigated whether fisetin induced apoptosis is related to the generation of ROS. For this, we employed the cell-permeable DCFH-DA, probe which in the presence of ROS is rapidly oxidized to highly fluorescent DCF, easily quantified on a fluorometric plate reader. Fisetin treatment to A375 melanoma cells resulted in inhibition of ROS generation at all time points studied, beginning from 30 min post treatment and ending at 24 h, signifying that fisetin induced apoptosis is not mediated through ROS generation (Fig.3A). Fisetin treatment to 451Lu cells did not result in any appreciable change in ROS levels (Fig.3B) however a marked increase in NO generation was evident with fisetin treatment particularly at extended time points (Fig.3D). This phenomenon was also reciprocated in fisetin-treated A375 cells (Fig.3C).

Apoptosis is accompanied with a transient autophagic response in fisetin treated melanoma cells

Time course analysis of fisetin treated A375 melanoma cells demonstrated that activation of extrinsic and intrinsic pathways occurred as early as 12 h post treatment (Fig.4A). To explore the possible involvement of autophagy in fisetin induced cell death, we determined the expression of the autophagy marker LC-3 in fisetin treated cells. LC-3, a mammalian homolog of yeast Atg8, undergoes characteristic lipidation during autophagosome formation yielding a lower mobility LC-3-I and a lipidated higher mobility LC-3-II (21). Fig.4A shows that treatment with fisetin resulted in accumulation of the LC-3-II form in A375 melanoma cells at 12 h. Notably, LC-3-II accumulation persisted in fisetin treated cells for 24-36 h but was absent at later time points, suggesting that induction of autophagy was a concomitant though transient effect in fisetin treated cells. Because monodansylcadaverine (MDC) accumulates in mature autophagic vacuoles, such as autophagolysosomes, we further confirmed fisetin induced autophagy by MDC-staining of A375 cells. A significant increase in MDC staining was observed in fisetin treated A375 cells as compared to the untreated control (Fig.4A, *bottom panel*). In contrast to A375 cells, fisetin induced autophagy occurred at 48h in 451Lu cells (Fig.4B; SFig.3). In the canonical autophagy pathway, Beclin-1 initiates the generation of the autophagosome by forming a multiprotein complex with class III phosphatidylinositol-3-kinase (PI3KIII) (21). Fig.4B shows a dose-dependent

increase in the protein expression of Beclin and ATG5 in fisetin treated 451Lu cells. Moreover, immunofluorescence staining showed punctate staining of LC-3-positive autophagic vesicles in 451Lu cells treated with fisetin for 48 h (Fig.4B, *bottom panel*). To examine if inhibition of fisetin induced autophagy can alter cell viability, we treated 451Lu cells with a widely used PI3KIII inhibitor 3MethylAdenine (3MA), and analyzed cell death by flowcytometry. Treatment with 3MA and fisetin for 48 h showed an increase in the percentage of cells undergoing apoptosis, suggesting a shift of autophagic cell death towards apoptosis (Fig.4C). Finally, we compared the mode of cell death in 3-D melanoma constructs treated with fisetin for 16 days every alternate day. In addition to a significant inhibition in melanoma growth, we observed intense staining of Caspase-3 in fisetin treated constructs (Fig.4D). This was in stark difference to LC-3 staining which showed no difference between the control and fisetin treated groups (SFig.4), suggesting that apoptosis is the primary mechanism of fisetin induced cell death.

Fisetin mediated apoptosis is associated with induction of ER stress in melanoma cells

ER stress is implicated in the induction of apoptosis as well as autophagy (22). To assess whether fisetin induced apoptosis is linked to ER stress, we evaluated the effect of the compound on ER stress markers. Activation of IRE1 promotes the splicing of a 26 nucleotide intron from the XBP1s mRNA to give rise to its spliced variant XBP1s (7). Treatment of A375 and 451Lu cells for 24 h with fisetin (20-80 μ M) showed a sustained increase in the levels of IRE1 and XBP1s in both cell lines (Fig.5A). We then proceeded to examine the other two arms (PERK/CHOP and the ATF6) of ER stress signaling. Immunoblot analysis of fisetin treated cells for 24 h demonstrated significant induction of ATF4 in both cell lines however the increase in the protein expressions of PERK, CHOP and ATF6 in A375 cells was not reflected in 451Lu cells (Fig.5A), suggesting that fisetin induced ER stress may be mediated through activation of different ER receptor pathways in different cell lines. mRNA studies in A375 melanoma cells showed increased transcript levels of XBP1s, ATF4 and GRP78 (Fig.5B). The upregulation of molecular chaperones including GRP78, Calnexin and the oxidoreductase ERO1 α sensitive to changes in the ER redox state, further confirmed the induction of ER stress in fisetin treated cells (Fig.5C). Severe ER stress leads to activation of c-Jun N-terminal kinase (JNK) and induction of CHOP, with subsequent neutralization of the anti-apoptotic effect of Bcl-2 (23). A time course analysis of fisetin treated A375 melanoma cells demonstrated that induction of ER stress markers corresponded to the phosphorylation and activation of JNK (Fig.5D). We further studied fisetin induced ER stress response in ESRE-*bla* HeLa cells engineered to express β -lactamase under the control of ER stress response element through the ATF6 receptor. Here we assessed the activation of ER stress response 5 h post fisetin treatment. Fisetin treatment did not induce the activation of β -lactamase reporter activity, measured quantitatively with the LiveBLAzerTM-FRET B/G loading substrate in these cells, indicating that fisetin induced stress response is time-dependent and different arms of the ER pathway may be activated in cell-type specific manner (Fig.5D).

Fisetin induced cytotoxicity is independent of AMPK activation

AMPK is activated by hormonal and nutrient stresses that deplete intracellular ATP levels and alter the AMP-ATP ratio. The activation of the enzyme adenylyl cyclase catalyzes the

conversion of ATP into cAMP (24). We investigated whether fisetin treatment by affecting cellular bioenergetics resulted in a decrease in ATP-cAMP ratio. To test this, we examined the changes in cellular ATP levels in response to fisetin treatment, employing the Cyclic AMP XP® Assay. Fig. 6A&B show the decrease in ATP levels in fisetin treated cells in a dose- and time-dependent manner. To determine if the increase in cAMP levels and the consequent decreased ATP levels was responsible for AMPK activation we examined the phosphorylation status of AMPK in fisetin treated cells. Fig. 6C shows that fisetin treatment induced significant phosphorylation of AMPK at the Thr¹⁷² residue indicating that activation of AMPK is linked to fisetin induced alterations in cellular energy levels. Another indicator of low cellular energy is phosphorylation of ACC which is directly regulated by AMPK. As shown in the Fig. 6D, increased phosphorylation of ACC was evident 12 h post fisetin treatment corresponding to AMPK phosphorylation and decreased ATP levels. Earlier reports suggested the involvement of AMPK in fisetin induced cell death (25). To scrutinize the role of AMPK in fisetin-induced cell death, we transfected A375 cells with siRNA against AMPK and examined the effect on apoptotic and autophagy markers. Unexpectedly, Western blot analysis showed that fisetin-induced apoptosis (measured by cleavage of Caspase-3 and PARP) and autophagy (measured by LC-3-II accumulation) were unaffected in AMPK suppressed cells treated with fisetin (Fig. 6E). Next, the ER stress markers were studied. Remarkably, AMPK silencing had no inhibitory effect on fisetin mediated upregulation of ER stress markers XBP1s, ATF4 and CHOP in A375 melanoma cells (Fig. 6F).

Discussion

Dietary agents are being investigated for their role in prevention and treatment of cancer and may represent the future modality of the treatment (20, 26). In this respect, naturally occurring agents that are mechanistically linked to events in melanoma carcinogenesis can be potential candidates for the prevention and treatment of this disease. We previously showed that the dietary flavonoid fisetin decreased Mitf levels in 451Lu melanoma cells through interference with Wnt/ β -catenin signaling and inhibited tumor growth in a xenograft model (20). Employing cell free systems and 3-D melanoma skin equivalent constructs, we next demonstrated, that fisetin binds with much higher affinity to p70S6K and mTOR in comparison to AKT, to execute its growth inhibitory effect on cancer cells (27). The aim of the current study was to further dissect the molecular and biochemical pathways activated in fisetin-induced cytotoxicity and subsequent growth inhibition in human melanoma cells.

Several chemotherapeutic agents are known to induce necrotic or apoptotic cell death in cancer cells through the generation of ROS (28). Conversely, many tumor cells exist in a pro-oxidant state that is not necessarily detrimental and does not equate with lethal oxidative stress (29, 30). In addition, physiological levels of some ROS, particularly O₂⁻ and H₂O₂, are required for critical cellular functions such as cell adhesion, immune functions and certain growth factor-dependent survival pathways (29, 31). In this context, it was shown that exposure of cells to superoxide dismutase mimetic for inhibition of O₂⁻ or the antioxidant *N*-acetyl-L-cysteine (NAC) to reduce ROS strongly enhanced etoposide-induced apoptosis (32). We observed that fisetin treatment to A375 melanoma cells resulted in a significant decrease in ROS levels (Fig. 3A). Our results contrast with those obtained by Jang

et al who demonstrated that fisetin stimulated generation of ROS in U266 multiple myeloma cells and induced apoptosis through activation of AMPK pathways in a ROS dependent manner (25). Parallel studies in other systems however, indicate that fisetin attenuates hydrogen peroxide-induced cell damage by scavenging ROS and activating protective functions of cellular glutathione system (33). Fisetin improved the antioxidant competence in hepatic tissues of diabetic rats and protected hepatocellular ultrastructure from hyperglycemia mediated oxidative stress (34). We speculate that lower ROS levels in fisetin treated cells may predispose to inhibited cell growth due to decreased signaling mediated by pro-survival pathways such as PI3K/AKT/ mTOR, or the NF-KB. This notion is corroborated from recent studies where it was shown that NAC in non-toxic concentrations can enhance fisetin-mediated apoptosis in colon cancer cells (35). Similar results were seen in A375 melanoma cells that were pre-incubated with NAC prior to treatment with fisetin (SFig.2). Interestingly, fisetin treatment did not result in a statistically significant decrease in ROS generation in 451Lu melanoma cells, at least at 24 h, indicating that modulation of ROS by fisetin can be cell type dependent. Fisetin potentiated the increase in NO especially at extended time points in both cell lines. Reactive nitrogen species have been implicated as possible regulators of autophagy through TSC2-mediated suppression of mTOR complex 1(36). Though further studies are needed to prove this line of thought, it is interesting to note that fisetin stabilizes the TSC complex and has been shown to inhibit mTORC1 signaling in various cancer cell models (37, 38).

Despite initial observations that the autophagy related protein Beclin-1 functioned as a tumor suppressor, various studies have indicated that autophagy may also function as a survival mechanism adopted by cancer cells facing hostile microenvironment (39, 40). Several currently used anticancer agents are known to induce the accumulation of autophagosomes *in vitro*, which likely represents a protective mechanism against the stress induced by cytotoxic agents (9, 41-43). In order to determine the role of autophagy in fisetin induced cell death we reverted to 3-D constructs which enabled us to monitor cellular response for an extended period. Studies conducted in 3-D melanoma model treated with fisetin for 16 days did not show any significant difference in LC-3 staining in control and treated groups at days 12 and 16 (SFig.4). Similar results were observed in archival tissues of fisetin-treated 451Lu xenografts (data not shown). Fisetin treatment to human melanoma cells resulted in a modest increase in cells undergoing apoptosis, when autophagy was downregulated through pharmacological inhibition (Fig.4C), supporting the notion that induction of autophagy in these cells represents an adaptive survival mechanism to fisetin-induced damage.

In line with its proposed role, AMPK activation in response to changes in cellular energy levels switches on the catabolic pathways that generate ATP while switching off anabolic pathways and other processes that consume ATP. Examples of this include acute inhibition of lipid biosynthesis by phosphorylation and inactivation of key metabolic enzymes such as ACC1 (fatty acid synthesis), glycerol phosphate acyl transferase (triacylglycerol synthesis), and HMG-CoA reductase (cholesterol/isoprenoid biosynthesis) (44). Our studies show that fisetin treatment resulted in phosphorylation and activation of AMPK and ACC, which was more significant at an early time point (Fig.6). The precise role of AMPK in fisetin induced

cell death remains ambiguous. Previous work had indicated that fisetin exerts its cytotoxic effects through the activation of AMPK pathway (25). Gene silencing studies demonstrating that inhibition of AMPK did not prevent cell death advocate an alternate role of AMPK in fisetin treated melanoma cells (Fig.6). The notion that AMPK acts solely as a tumor suppressor conflicts with reports that show that it confers resistance to nutrient deprivation, sustains NADPH levels in cancer cells, facilitates stress-induced gene transcription, promotes cell survival and increases malignant transformation (45, 46). It seems plausible that fisetin mediated activation of AMPK may be part of a multi-pronged defense mechanism mounted by the cellular machinery subsequent to alterations in cellular energy levels.

Any stress which negatively impacts upon energy availability or intracellular calcium levels can trigger ER stress (23). The balance between the amount of stress and the cell capacity to cope with insults then determines the outcome: cell survival or death. Activation of the intrinsic apoptotic pathway as a result of unresolved ER stress occurs through members of the Bcl-2 family which control the release of cytochrome *c* from the mitochondria. Recent reports have identified the ER stress sensor GRP78 as a useful therapeutic target to enhance tumor cell death (47). JNK-mediated phosphorylation of Bcl-2/Bcl-xL has been reported to decrease the anti-apoptotic ability while phosphorylation of Bid and Bim by JNK increases the apoptotic ability of the cells (23). In our studies, the effect of fisetin on the three arms of ER stress regulators varied somewhat with the cell type, so that in 451Lu cells we did not observe an upregulation of PERK, evident in A375 cells. Additionally studies in Hela cells overexpressing the ATF6 promoter did not show induction of ER stress with fisetin treatment suggesting regulation of ER stress may occur through alternate pathways dependent on the cellular context. Nonetheless, it is clear from our data that fisetin upregulated GRP78 and induced ER stress in both cell lines, albeit through modulation of different arms of the stress pathway associated with induction of apoptosis in melanoma cells. In our time-dependent studies we found increased JNK phosphorylation correlating to activation of IRE1 α in fisetin treated melanoma cells. Furthermore, decrease in Bcl-2 (Fig. 2B) and Bcl-xL (data not shown) and upregulation of Bim (SFig.3) subsequent to fisetin treatment suggests that fisetin mediated activation of IRE1 α /JNK pathway may represent a possible mechanism through which the ER-stress balance may tip in favor of apoptosis.

Taken together, our data show that fisetin triggered caspase-dependent cell death in melanoma cells and these cells activated autophagy as a survival mechanism against fisetin induced bioenergetics failure. ER stress and AMPK activation play an important role in fisetin induced cytotoxicity through opposing effects on cell survival. This study provides further evidence that the flavonoid fisetin has multiple effects in cancer cells which can be exploited alone or in conjunction with other therapeutic regimens to prevent melanoma growth.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by United States Public Health Service Grants R01CA160867 and T32 ES007015. The authors thank Brendan T. Boylan for his assistance in 3-D immunostaining.

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Figure 1. Fisetin induces apoptosis in melanoma cells

(A) Representative micrographs of fisetin treated A375 cells (20×) undergoing apoptosis as assessed by fluorescence microscopy. Cells were treated with vehicle alone or fisetin (60μM; 24 h). Apoptosis and necrosis were detected by Nikon Eclipse Ti fluorescent microscope as described in materials and methods. (B) A375 and 451Lu cells treated with fisetin (20–80 μM; 48 h) were stained with PI/FITC using the Apo-Direct kit as per vendor's protocol. Following FACS analysis, cellular DNA histograms were analyzed by ModifiLT V3.0. % age apoptosis is given for each sample in the figure. The data are representative of duplicate tests.

Figure 2. Fisetin induces apoptosis through activation of the extrinsic and intrinsic apoptotic pathways in melanoma cells

(A&B) Effect of fisetin on the protein expression of death receptors TNFR-1 and TNFR-2, components of the extrinsic pathway (A) apoptosis related proteins, Bax and B-cl2, components of the intrinsic pathway (B): Whole cell lysates of A375/451Lu cells treated with/without fisetin (20–80 μM, 24h) were subjected to SDS-polyacrylamide gel electrophoresis. Equal loading was confirmed by reprobing for -actin. The immunoblots shown are representative of three independent experiments with similar results. (C&D) Effect of fisetin on Caspases 8 and 9 activities in fisetin-treated cells: 451Lu cells were incubated in the absence or presence of fisetin (60 μM: 24 h) and the whole-cell lysate were subjected to Caspase-8 and Caspase-9 activity assays, as per manufacturer's protocol. The data expressed as the percentage enrichment factor represent the mean±standard errors of experiments done in replicates where $p < 0.05$ was considered significant. (E) Effect of fisetin on Caspase-3: Representative micrographs (40x) of A375/451Lu cells treated with/without fisetin (60μM: 24 h). Immunocytochemical analysis show intense Caspase-3 staining in treated cells as opposed to the untreated controls. (F) Effect of fisetin on PARP cleavage: Whole cell lysates of A375/451Lu cells treated with fisetin (20–80 μM, 24h) were subjected to SDS-polyacrylamide gel electrophoresis. Equal loading was confirmed by

reprobing for α -actin. The immunoblots shown are representative of three independent experiments with similar results.

Figure 3. Fisetin inhibits ROS and augments NO in melanoma cells

(A&B) Fisetin inhibits ROS generation in A375 melanoma cells but has no significant effect on 451Lu melanoma cells: Intracellular levels of ROS as a function of exposure to fisetin treatment (60 μ M) to A375 (A) and 451Lu (B) melanoma cells was determined at varying time points. Post treatment at specified time points the fluorescence was read on a standard plate reader at 480 nm excitation/ 530 nm emission. Mean fluorescent intensity value is proportional to intracellular levels of ROS. (C&D) Fisetin augments NO in melanoma cells: Intracellular levels of NO as a function of exposure to fisetin treatment (60 μ M) to A375 (C) and 451Lu (D) melanoma cells was determined at varying time points. Post treatment at specified time points the fluorescence was read on a standard plate reader at 480 nm excitation/ 530 nm emission. Mean fluorescent intensity value is proportional to intracellular levels of NO. The data presented represent the mean $\pm$ SD of experiments done in replicated, where $p < 0.05$ was considered significant.

Fig. 4. Apoptosis is accompanied with a transient autophagic response in fisetin treated melanoma cells

(A) Fisetin induces transient increase in the expression of autophagy marker LC-3 in A375 melanoma cells: Time course analysis of fisetin treated A375 cells (60 μ M) for markers of apoptosis and autophagy. Equal loading was confirmed by reprobing for β -actin. Data shown are representative of three independent experiments (*top panel*). MDC fluorescent intensity of fisetin-treated A375 cells examined by confocal microscopy (10 $\times$). The results are representative of at least two different experiments (*bottom panel*). (B) Fisetin induces autophagy in 451Lu melanoma cells: Whole cell lysates of fisetin treated 451Lu melanoma cells (48 h) were analyzed by western blot analysis for Beclin and ATG5 protein expression. Equal loading was confirmed by reprobing for β -actin. Data shown are representative of two independent experiments (*top panel*). 451Lu cells seeded on tissue culture slides and treated with/without fisetin, fixed in 2% paraformaldehyde, and incubated with LC-3 anti-antibody were observed under the Nikon Eclipse Ti microscope (10 $\times$). 4,6-Diamidino-2-phenylindole (DAPI) was used to counter stain the nucleus (*bottom panel*). (C) Inhibition of autophagy increases the percentage of cells undergoing apoptosis: 451Lu cells cotreated with PI3K inhibitor 3MA (1 mM) and fisetin (60 μ M) for 48 h were collected and stained with PI using the Apo-Direct kit. Following FACS analysis, cellular DNA histograms were analyzed by

ModiFitLT V3.0. Top panel shows representative micrographs of 451Lu cells treated with fisetin with or without 3MA (D) Fisetin induces apoptosis associated with tumors inhibition in a 3-D melanoma model. Representative photomicrographs (10×) showing immunohistochemical staining for Caspase-3 in A375 melanoma constructs treated with fisetin (80 μ M) , harvested at days 12 and 16 post treatment.

Fig. 5. Fisetin mediated apoptosis is associated with induction of ER stress in melanoma cells (A-C) Fisetin induces ER stress markers: (A) A375 and 451Lu cells were treated with varying doses of fisetin for 24 h and western blot analysis was performed. Equal loading was confirmed by reprobing for β -actin. Data shown are representative of three independent experiments. (B) A375 cells treated with fisetin (40 μ M; 24 h) were analyzed for changes in mRNA levels of XBP1s, ATF4 and GRP78, using qPCR. The data expressed as fold change represent the mean $\pm$ standard errors experiments performed in triplicates where $p < 0.05$ was considered significant. (C) A375 and 451Lu cells were treated with varying doses of fisetin for 24 h and western blot analysis was performed for ER stress markers. Equal loading was confirmed by reprobing for β -actin. Data shown are representative of three independent experiments. (D) Fisetin induced ER stress occurs 12 h post fisetin treatment and ER stress pathways are cell type specific: Time course analysis of fisetin treated A375 cells (60 μ M; 24 h) for cellular stress markers. Equal loading was confirmed by reprobing for β -actin. Data shown are representative of three independent experiments (*top panel*). ER-Activator assay: ESRE-bla HeLa cells are engineered to express β -lactamase under the control of ER stress response element through the ATF6 receptor treated with fisetin for 5 h (*bottom panel*).

Fig. 6. Fisetin induced cytotoxicity is independent of AMPK activation

(A&B) Fisetin decreases ATP levels: 451Lu cells treated with Fisetin at different doses (A) and times (h) (B) were analyzed for cAMP/ATP levels using Cyclic AMP XPT™ Assay kit. Asterisks indicate statistically significant change vs. corresponding control where $p < 0.05$ was considered significant. (C&D) Fisetin activates AMPK signaling: (C) Whole cell lysates of Fisetin treated 451Lu cells (40–80 μ M, 24 h) were analyzed by Western blot analysis for phosphorylated and total AMPK. Equal loading was confirmed by reprobing for β -actin. Data shown are representative of three independent experiments. (D) Time course analysis of Fisetin treated A375 cells (60 μ M: 24 h) for phosphorylated AMPK and ACC. Equal loading was confirmed by reprobing for β -actin. Data shown are representative of three independent experiments. (E) Fisetin induced cytotoxicity is independent of AMPK activation: A375 cells transfected with siAMPK and treated with Fisetin (60 μ M: 24 h) were analyzed for apoptosis and autophagy markers PARP, Caspase-3 and LC-3 respectively. Equal loading was confirmed by reprobing for β -actin. Data shown are representative of at least two independent experiments. (F) Silencing of AMPK had no effect on Fisetin induced ER stress markers: A375 cells transfected with siAMPK and treated with Fisetin (60 μ M: 24 h) were analyzed for ER stress markers XBP1s, ATF4 and CHOP. Equal loading was

confirmed by reprobing for β -actin. Data shown are representative of at least two independent experiments.