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Exploring the molecular targets of dietary flavonoid fisetin in cancer

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Abstract

The last few decades have seen a resurgence of interest among the scientific community in exploring the efficacy of natural compounds against various human cancers. Compounds of plant origin belonging to different groups such as alkaloids, flavonoids and polyphenols evaluated for their cancer preventive effects have yielded promising data, thereby offering a potential therapeutic alternative against this deadly disease. The flavonol fisetin (3,3',4',7-tetrahydroxyflavone), present in fruits and vegetables such as strawberries, apple, cucumber, persimmon, grape and onion, was shown to possess anti-microbial, anti-inflammatory, anti-oxidant and more significantly anti-carcinogenic activity when assessed in diverse cell culture and animal model systems. The purpose of this review is to update and discuss key findings obtained till date from *in vitro* and *in vivo* studies on fisetin, with special focus on its anti-cancer role. The molecular mechanism(s) described in the observed growth inhibitory effects of fisetin in different cancer cell types is also summarized. Moreover, an attempt is made to analyze the direction required for future studies that could lead to the development of fisetin as a potent chemopreventive/chemotherapeutic agent against cancer.

Keywords

Flavonoid; Fisetin; Bioavailability; Anti-oxidant; Cancer; Apoptosis; Autophagy; Migration; Invasion

1. Introduction

The flavonoids belong to a large family of plant-derived compounds containing more than 4000 secondary plant metabolites which exhibit several biological effects. The four major classes of flavonoids include 4-oxoflavonoids (flavones and flavonols), isoflavones,

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Conflict of interest

The authors declare that there are no conflicts of interest.

anthocyanins, and flavan-3-ol derivatives (tannins and catechin) [1]. It is essential to identify the sources of flavonoids in food and to study their effects as amount of flavonoids in diet is directly related to the dietary consumption of antioxidants. Fisetin (3,3',4',7-tetrahydroxyflavone) is mostly present in food-based products such as apples, strawberries, cucumber and onions [2] and has been reported to exert pleiotropic effects in different disease models, both *in vitro* and *in vivo* [3–8]. In this review, we have attempted to summarize the literature available on the anti-cancer effects of the dietary flavonoid fisetin with special emphasis on its role in cellular processes including cell death, growth and proliferation.

2. Bioavailability and pharmacokinetics of fisetin

Despite considerable amount of data on the biological activities of fisetin, only a limited number of studies have been performed to assess its bioavailability in body tissues. Shia et al. [9] investigated the metabolism and pharmacokinetics of fisetin in male Sprague-Dawley rats. After an intravenous (iv) dose of fisetin (10 mg/kg body weight), there was a rapid decline of fisetin concomitant with the appearance of sulfate and glucuronide conjugates of fisetin. However, upon oral administration of fisetin (50 mg/kg of body weight), presence of fisetin was detected albeit briefly in serum specifically in the absorption phase followed by an increase in fisetin sulfates/glucuronides. The serum metabolites of fisetin were found to be less effective against 2,2'-azobis(2-amidinopropane hydrochloride)-induced hemolysis as compared with fisetin [9]. Comparative studies with flavones such as 5-OH-flavone and 7-OH-flavone indicated that fisetin and 7-OH-flavone were rapidly biotransformed into their respective sulfate or glucuronide metabolites while 5-OH-flavone was exclusively metabolized to glucuronides [9]. Thus, the number and position of the hydroxyl (OH) group as well as the charge on the flavone structure may be an important determinant of the substrate toward glucuronidation or sulfation. Although the three compounds were administered as clear solutions at the same molar dose, it appeared that 5-OH-flavone and 7-OH-flavone were markedly less bioavailable than fisetin. It was speculated that the presence of four phenolic groups in fisetin may account for its greater solubility and better absorption. Moreover, transient saturation of the conjugation metabolism due to its greater bioavailability may also explain the presence of the parent form of fisetin during the absorption phase [9].

Touil et al. [10] determined the pharmacokinetics and metabolism of fisetin in mice and studied the biological activities of its metabolites. Their studies showed that after an intraperitoneal (ip) dose of 223 mg/kg body weight the maximum plasma concentration (2.53 µg/ml) of fisetin was reached at 15 min which started to decline with a first rapid alpha half-life of 0.09 h and a longer half-life of 3.12 h. Three metabolites of fisetin were detected including the methoxylated metabolite geraldol. The latter was shown to achieve higher concentrations than fisetin in tumor-bearing mice and appeared more cytotoxic than the parent compound [10]. Bioavailability studies further indicated that the plasma concentrations of fisetin in mice were higher than those noted in rats where insignificant levels of free fisetin and a short half-life of 2.7 min was observed. Additional experiments are needed to determine whether slower elimination of fisetin in mice due to lower conjugation capacity through glucuronidation and an enhanced retention time contributes to

greater efficacy. The presence of varied metabolites in different species makes it difficult to infer the precise effect of the compound and relate it to its reported *in vitro* activities. The principal metabolites detected in animal studies possess different physicochemical properties from the parent compound and can exert a more potent effect in an *in vivo* setting [9]. Finally due to restricted availability of the metabolites, most *in vitro* bioactivity studies of flavonoids have focused on the parent compounds and different formulations are being currently investigated.

Since limited water solubility (<1 mg/ml) of fisetin was recognized as a constraint for its therapeutic efficacy in animal disease models, efforts were directed to increase the bioavailability through various formulations [11]. Bothiraja et al. [12] examined the bioavailability of fisetin-loaded nanocochelates which are lipid-based supramolecular assemblies containing negatively charged phospholipid and a divalent cation. Pharmacokinetic studies in mice showed that there was a sustained release of fisetin at physiological pH and when nanocochleates were administered ip, there was low tissue distribution and massive increase (141-fold) in relative bioavailability [12]. In another study, the complexation of fisetin with cyclophorase (Cys) dimer, an exopolysaccharide produced by many species of the *Rhizobiaceae* family, composed of unbranched cyclic oligosaccharides joined by glucose units through β -(1,2)-linkages, was investigated to improve the solubility of fisetin [13]. The solubility of fisetin was improved 6.5-times after complexation with Cys dimer and this was 2.4-times better than with β -cyclodextrin. Increased cytotoxicity to HeLa cells by the fisetin-Cys dimer complex than free fisetin suggested that Cys dimer increased bioavailability of fisetin [13].

The effect of the liposomal formulation on the bioavailability fisetin was studied in Lewis lung carcinoma bearing mice [14]. The pharmacokinetics of free and liposomal fisetin administered through the iv or ip mode of administration were compared (Table 1). The assessment of free fisetin with liposomal fisetin given at a dose of 13 mg/kg iv revealed that the liposomal formulation had a modest benefit in systemic exposure [14]. Upon ip administration of the liposomal formulation of fisetin (21 mg/kg body weight) and free fisetin (223 mg/kg body weight), it was found that the liposomal fisetin produced higher fisetin plasma concentrations; even though the dose was 10 times less than that of free fisetin. The calculated relative bioavailability was reported to be 47-times higher for liposomal fisetin as compared with free fisetin [14]. The concentrations of fisetin were further studied in major organs 15 min after iv dose of 13 mg/kg of free fisetin or its liposomal form. The concentrations of liposomal fisetin were five times higher in liver while twice that of free fisetin in the blood. Lungs, kidneys, spleens and the tumors did not display any significant difference in fisetin concentration [14].

To attain better bioavailability so that fisetin could be suitable for parenteral administration, it was formulated into nanoemulsion and the pharmacokinetics studies were performed in mice after iv or ip treatments [15]. Relative pharmacokinetic profiles were studied when free fisetin formulation or its nanoemulsion at a dose of 13 mg/kg was injected iv in mice (Table 1). Treatment with fisetin nanoemulsion with a dose half that of free fisetin, caused a significant elevation in plasma concentrations of fisetin and 24-times higher relative bioavailability as compared to free fisetin. This might be due to the fact that nanoemulsion is

more rapidly absorbed with a shorter mean absorption time of 2 h as compared with 6 h for the free fisetin [14]. Thus, metabolism seems to be an important determinant of the biological responses and anticancer properties of fisetin. Importantly in the absence of well-designed studies in humans, it remains to be seen whether the biological activities of fisetin observed *in vitro* and in animal studies can be extended to human subjects.

3. Anti-oxidant activity of fisetin

Generation of oxygen radicals has been associated with the development and progression of many age related diseases, including diabetes mellitus, retinal degeneration, neurodegenerative disorders, mutagenesis, ageing as well as carcinogenesis [16]. Various studies have reported that dietary agents, including fisetin, act as promising antioxidants by playing a significant part in the prevention/therapy of illnesses triggered by oxidative stress [8]. Analysis of OH bond dissociation energy and dipole moment suggested that fisetin possesses high antioxidant capacity [17] as evidenced by its high trolox-equivalent activity concentration (TEAC) value i.e. 2.80 – 0.06 [18]. This high antioxidant capacity of fisetin was further confirmed by semiempirical calculations for fisetin. The calculated lowest bond dissociation enthalpies (BDE) value was for the 3-OH fisetin radical, which was followed by the 3'-OH and 4'-OH fisetin radicals. The highest BDE value was predicted for 7-OH radical. A lower BDE value is credited to a greater capacity to donate a hydrogen atom from the OH group and thus scavenge free radicals. Therefore, this study showed that the OH groups in the 3, 3', 4' positions of fisetin were more effective in scavenging free radicals than the 7-OH group on the A-ring [19]. Fisetin showed the lowest oxidation potential in a ferric reducing antioxidant assay, which directly determines the reducing capacity of a compound. Structure based analysis suggested that the o-dihydroxy structure in the B ring, 3-hydroxy group and 2,3-double bond in the C ring mainly contribute to the antioxidant activity of fisetin [20]. In a comparative study using Cu²⁺⁺ mediated LDL oxidation as a read out, it was found that fisetin holds a stronger oxidant inhibitory activity than well-known potent antioxidants like morin and myricetin. *Ex vivo* studies using primary rat neurons showed that fisetin effectively protected against SIN-1 mediated alterations in inducing extracellular signal-regulated kinase1/2 (ERK)/c-myc phosphorylation, nuclear NF-E2-related factor-2 (Nrf2), glutamate cystine ligase and glutathione (GSH) levels [21]. Similarly, increase in intracellular GSH levels was also recorded in mouse hippocampal HT-22 cells when treated with fisetin [22]. Treatment of umbilical vein endothelial cells with fisetin activated Nrf2 and its nuclear translocation [23]. Also, fisetin was found to activate Nrf2 mediated induction of hemeoxygenase-1 (HO-1) important for cell survival under oxidative stress conditions [23]. More recent studies have found that fisetin attenuates oxidant-driven activation of c-Jun N-terminal protein kinase (JNK) and nuclear factor-kappa B (NF-κB) signaling pathways [24,25]. These data suggest a possible use of fisetin as a potent anti-oxidant in a wide variety of biological conditions.

4. Anti-cancer activity of fisetin

4.1. Fisetin and cell proliferation

In addition to its anti-oxidant activity, we [3,5,26–31] and others [4,7] have extensively shown the potential of fisetin in affecting signaling pathways that control cell survival, growth and proliferation both *in vitro* and *in vivo*. Haddad et al. [7] observed a decrease in proliferation with concomitant induction of apoptosis in prostate cancer cells upon fisetin treatment. Similar studies from our laboratory showed that fisetin decreased proliferation and growth of both androgen dependent and independent prostate cancer cells namely LNCaP, CWR22Rv1 and PC-3 cells but had minimal effect on normal prostate epithelial cells [3]. Fisetin was shown to interact with the ligand binding domain of the androgen receptor (AR), and its interference with the amino-/carboxyl-terminal interaction was found to blunt AR-mediated transactivation of target genes [27]. Treatment with fisetin in athymic nude mice implanted with AR-positive CWR22Rv1 cells resulted in inhibition of tumor growth associated with reduction in serum PSA levels [27]. In our *in vitro* studies, we further observed that fisetin inhibited mammalian target of rapamycin (mTOR) complexes 1 and 2 and suppressed Cap-dependent translation [28,29]. In our studies on fisetin in non-small lung cancer cells, we found that fisetin acts as a dual inhibitor PI3K/Akt and mTOR pathways [30]. This appears to be an exciting observation since both Akt and mTOR pathways are among the major signaling networks that have been implicated in advanced cancer [29]. Using *in silico* modeling we showed that fisetin interacts with mTOR at two sites, thereby explaining its inhibitory effect on cellular growth and proliferation [30]. Fisetin-treated cells exhibited dose-dependent inhibition of the constituents of mTOR signaling complex such as Rictor, Raptor, GβL and PRAS40 [30]. We had reported previously that fisetin negatively regulated the growth of human melanoma cells through disruption of Wnt/β-catenin signaling and decreased Mitf levels [31]. We extended these studies in melanoma cells and evaluated the relative binding affinities of fisetin to kinases involved in growth and proliferation [32]. Our studies in melanoma monolayers and 3D melanoma skin equivalent model demonstrated that fisetin targets p70S6K and mTOR (Figure 1) and exerts an inhibitory effect on the growth of human melanoma cells through direct binding to these kinases [32]. Interestingly, fisetin was found to have a very low binding affinity to Akt suggesting that the decreased phosphorylation of Akt observed upon fisetin treatment was mediated through its effect on interrelated pathways [32]. Recently, fisetin was shown to inhibit growth and proliferation of human melanoma cells both *in vitro* and *in vivo* in combination with the BRAF inhibitor sorafenib [33]. Fisetin has also shown strong negative impact on the growth and proliferation of diverse cancer cell types including breast [34–36] cervical [37,38] and colon [39–43].

4.2. Fisetin and cell cycle

With increased understanding of the process of carcinogenesis, the role of cell cycle in malignant transformation and disease progression cannot be overrated. Thus, cell cycle regulatory molecules are deemed rational therapeutic targets and several drugs targeting the cell cycle have entered clinical trials in cancer patients [44]. Gene expression profile of fisetin-treated PC-3 and LNCaP prostate cancer cells demonstrated that cell cycle regulatory genes were amongst the most highly represented functional categories of genes altered. Of

the 100 cell cycle genes modulated upon fisetin treatment, down-regulation of at least 27 genes involved in key functions in G2/M phase was observed [7].

The cell cycle profile of fisetin treated cells differs depending on cell type. A study in 3T3-L1 preadipocyte cell line showed that fisetin inhibited differentiation of adipocytes and proliferation of preadipocytes, accompanied by changes in expression levels of cell cycle regulatory proteins. It was suggested that inhibition of adipocyte differentiation may in part be mediated by fisetin induced cell cycle arrest during adipogenesis [45]. Our laboratory has shown that fisetin treatment to LNCaP cells resulted in G1-phase arrest accompanied with decrease in cyclins D1, D2 and E and their activating partner CDKs 2, 4 and 6 with induction of WAF1/p21 and KIP1/p27 [3]. In a screening study where flavonoids were examined for their effects on the cell cycle of prostate cancer cells fisetin was shown to induce G2/M phase arrest in PC-3 cells whereas LNCaP cells were arrested in both G1 and G2/M phases [46].

Fisetin induced G2/M arrest in human epidermoid carcinoma A431 cells while fisetin-treated 451Lu melanoma cells exhibited decreased viability with G1-phase arrest and disruption of Wnt/ β -catenin signaling [6,31]. In the HT-29 colon cancer cells, perturbed cell cycle progression from the G1 to S phase was observed within 8 h of fisetin treatment while the cells went into a G2/M phase arrest after 24 h. The phosphorylation state of the retinoblastoma proteins shifted from hyper-phosphorylated to hypo-phosphorylated. Fisetin-treated cells exhibited decreased protein levels of CDK1 and its upstream regulator CDC25C. Fisetin increased WAF1/p21 levels, suppressed cyclins E and D1 and inhibited the activities of CDK2 and CDK4. It was speculated that inhibition of cell cycle progression in HT-29 colon cancer cells after treatment with fisetin can be explained, at least in part, by its effect on CDKs [47].

The interaction of fisetin with CDK6 was examined in crystallography studies. Fisetin co-crystallized with CDK6 was shown to inhibit the activity of kinase. Fisetin formed hydrogen bonds with the side chains of residues in the binding pocket of CDK6 that undergo large conformational changes during CDK activation by cyclin binding. The 4-keto group and the 3-OH group of fisetin are hydrogen bonded with the backbone in the hinge region between the N- and C-termini of the kinase, as has been observed for other CDK inhibitors [48]. In another comparative molecular dynamics simulation study, the predicted inhibitory affinities were of the order of fisetin>apigenin>chrysin, against the CDK6/cyclinD complex [49]. It was shown that chrysin preferentially bound to the active CDK6 in a different orientation to fisetin and apigenin but similar to its related analog, deschloro-flavopiridol. A conserved interaction between the 4-keto group of the flavonoid and the backbone V101 nitrogen of CDK6 was observed for all three flavonoids. It was noted that the 3'- and 4'-OH groups on the flavonoid phenyl ring and the 3-OH group on the benzopyranone ring significantly increased the binding efficiency. In addition to the electrostatic interactions, especially through hydrogen bond formation, the van der Waals interactions with the I19, V27, F98, H100, and L152 residues of CDK6 were found to be crucial for the binding efficiency of flavonoids with the CDK6/cyclinD complex [49]. The obtained results provide useful information of the affinity and specificity of fisetin to cell cycle regulatory molecules which needs to be analyzed further for better exploitation of its anti-cancer effects.

4.3. Fisetin and microtubule assembly

Microtubules are an essential component of the cellular skeleton serving as the structural framework for various cellular processes including, but not limited to, cell division and motility, intracellular trafficking and cell shape [50]. In view of the great success of the anti-mitotic agents in the treatment of cancer, search for newer and safer microtubule-targeting agents is being earnestly investigated. This class of drugs is expected to continue to be important in the management of cancer in the future [51].

Using a cell-based high-throughput screen Salmela et al. [52] identified fisetin as an antimitotic compound. Fisetin treatment of several human cell lines compromised microtubule drug-induced mitotic block, caused premature initiation of chromosome segregation and exit from mitosis without normal cytokinesis. As a mechanism for these mitotic errors, Aurora B kinase was identified as a direct target of fisetin since Aurora B activity was significantly reduced by fisetin treatment [52]. Effects of fisetin on chromosome damage were investigated by Gollapudi et al. [53] and compared with two known Aurora kinase inhibitors, VX-680 and ZM-447439. Fisetin treatment of human lymphoblastoid TK6 cells resulted in induction of aneuploidy and polyploidy as indicated by increase in kinetochore-positive micronuclei, hyperdiploidy, and polyploidy. These observations suggested that fisetin could induce multiple types of chromosomal abnormalities in human cells and warranted caution in the use of fisetin containing products [53].

In this context, Touil et al. [54] reported fisetin as the most active microtubule stabilizer in a comparison study of twenty four flavonoids. Fisetin treatment induced rapid morphological modification of endothelial cells which correlated with an increase in microtubule stability and acetylation of α -tubulin, a marker of tubulin stabilization. Endothelial cells treated with fisetin showed resistance to cold induced depolymerization and a 2.4-fold increase in acetylated α -tubulin [54]. Fisetin treatment increased cellular asymmetry with numerous large extensions and filopodias indicating that fisetin affects both microtubule and actin filament organization [54].

We conducted detailed investigation into the effects of fisetin on microtubule dynamics based on our initial observation that fisetin enhanced tubulin polymerization, kinetics of which was far superior to that of the routinely used chemotherapeutic drug paclitaxel [55]. At similar doses the maximal velocity (V_{max}) for fisetin (65 m OD/min) was significantly higher than that of paclitaxel (12 m OD/min). We confirmed observations made by Touil et al. [54] using prostate cancer cells and showed that fisetin-treated cells are highly resistant to cold-induced microtubule depolymerization and that fisetin increases α -tubulin acetylation establishing its function as a microtubules stabilizer. Fisetin treated cells exhibited increased expression of MAP-2 and MAP-4 and reduced expression of nuclear migration protein Nud C. We found that subsequent to microtubule stabilization fisetin treated cells underwent G2/M phase arrest and subsequent cell growth inhibition [55].

Based on the assumption that fisetin directly interacts with microtubules, we conducted binding studies using surface plasmon resonance assays and observed that fisetin binds to β -tubulin, with a higher affinity ($KD:1.59 \mu M$) with respect to paclitaxel ($KD:2.26 \mu M$). In additional experiments we characterized the assumed interaction of fisetin with β -tubulin

using an *in silico* molecular docking approach. We examined the taxoid pocket within the β -tubulin molecule where paclitaxel binds and observed that fisetin binds within the same pocket albeit at a different orientation. Energy scoring poses and calculated free binding energy suggested various binding modes and tight affinity of fisetin within the taxoid pocket [55]. An important observation from the binding studies was that paclitaxel and fisetin do not compete and displace each other while bound within the same taxoid pocket.

Recent studies suggest failure of taxols in reducing tumor burden in advanced cancers [56,57]. Because fisetin interferes with many oncogenic cell signaling pathways in addition to its effects on microtubules it seems plausible that it could enhance the efficacy of conventional chemotherapeutic drugs in advanced and chemoresistant cancer cells. We made similar observations when fisetin was combined with anti-mitotic taxol cabazitaxel and tested against several prostate cancer cells and a chemoresistant cell line. Treatment of cells with a combination of fisetin and cabazitaxel significantly retarded the growth of prostate cancer cells PC-3, C4-2, 22Rv1 and a multidrug-resistant NCI/ADR-RES cell line when compared to vehicle, cabazitaxel or fisetin alone treated cells. When tested *in vivo* we observed that treatment with fisetin alone resulted in 14% inhibition of tumor growth; cabazitaxel treatment alone resulted in 36% inhibition whereas combination treatment with fisetin and cabazitaxel resulted in 53% inhibition of tumor growth [58,59]. Tissue staining with proliferation marker Ki67 confirmed the enhanced effect of the combination on inhibition of cell proliferation [59]. These findings highlight a potential use of a novel combination and provide evidence that activation of microtubule-stabilizing proteins could suppress cell proliferation and may interfere with cell migration and invasion.

4.4. Fisetin and cell migration and invasion

Initial studies exploring the effect of fisetin on prostate cancer demonstrated that fisetin could inhibit the metastatic ability of PC-3 cells by suppressing of PI3K/Akt and JNK signaling pathways with subsequent repression of matrix metalloproteinase-2 (MMP-2) and MMP-9 [60]. Involvement of ERK signaling has been reported in fisetin mediated inhibition of invasion and migration in the human lung cancer cell line A549. The study showed that fisetin suppressed protein and mRNA levels of MMP-2 and urokinase-type plasminogen activator (uPA) in an ERK-dependent fashion. A significant decrease in the nuclear levels of NF- κ B, c-Fos, and c-Jun was noted in fisetin treated cells [61]. Similarly, in glioma GBM8401 cells fisetin treatment resulted in sustained phosphorylation and activation of ERK1/2 with subsequent inhibition of ADAM9, a metalloproteinase involved in cell migration and invasion [62]. Fisetin repressed uPA in human cervical cancer cells via interruption of p38 MAPK-dependent NF- κ B signaling pathway. However, in contrast to earlier studies, it was demonstrated that fisetin reduced the phosphorylation of p38 MAPK, but had negligible effect on ERK1/2, JNK1/2, or Akt. Moreover suppression of tetradecanoylphorbol-13-acetate-mediated activation of p38 MAPK and reduced expression and secretion of uPA was observed in fisetin treated cells [37].

4.5. Fisetin and epithelial to mesenchymal transition (EMT)

The metastatic spread of cancer cells is understood to initiate by the reactivation of an evolutionary conserved developmental program known as EMT. During the course of EMT

fully differentiated epithelial cells undergo a series of changes in their morphology, along with loss of cell-to-cell contact and matrix remodeling to be converted into poorly differentiated, migratory and invasive mesenchymal cells [63]. In this context, the Epstein-Barr virus latent membrane protein-1 (LMP1) has been reported to induce EMT and is associated with metastasis of nasopharyngeal carcinoma cells. The impact of fisetin in preventing the migration and invasion of LMP1-expressing cancer cells and the molecular changes associated with LMP1 induced EMT were examined. The investigation demonstrated that fisetin suppressed the migration and invasion of CNE1-LMP1 cells under non-cytotoxic concentrations. Fisetin up-regulated the epithelial marker, E-cadherin and down-regulated the mesenchymal marker, vimentin accompanied by significant reduction in the levels of the EMT regulator Twist protein [64].

Efforts made to therapeutically target signaling molecules that govern the EMT program remain a challenge and have met with limited success. In a recent work we showed that fisetin inhibited EMT in two widely accepted models of EMT mainly induced by epidermal growth factor and transforming growth factor- β (TGF- β). We first treated the normal prostate epithelial cells with TGF- β to establish EMT followed by treatment with fisetin. Fisetin successfully reversed the morphology of prostate cells from mesenchymal to epithelial, evident by upregulation of E-cadherin and down-regulation of vimentin and N-cadherin. In addition, we also discovered that fisetin directly interacts and inhibits the nuclear translocation of the EMT inducing transcription factor Y box-1 (YB-1) [65]. Computational docking and dynamics study suggested that fisetin binds on the residues of β 1- β 4 strands of cold shock domain, hindering the interaction of Akt with YB-1 and resulting in inhibition of YB-1 driven EMT program [65].

Recently, the EMT inhibiting potential of fisetin has been reported in melanoma cells [33]. Fisetin was found to reduce human melanoma cell invasion by inhibiting N-cadherin, vimentin and fibronectin and inducing E-cadherin both *in vitro* and in xenografted tumors [33]. Finally, bioluminescent imaging of athymic mice, injected with stably transfected EGFP-tagged A375 melanoma cells demonstrated fewer lung metastases in mice treated with a combination of fisetin and the BRAF inhibitor sorafenib [33]. The impact of fisetin on EMT and processes mediated by EMT has only begun to be explored. Future studies will provide an in-depth analysis of the effect of fisetin on EMT-driven pathways.

4.6. Fisetin and cell death

4.6.1. Induction of apoptosis by fisetin—Apoptosis, a form of programmed cell death executed by activated caspases, can be exploited by anti-cancer drugs to prevent disease progression. In addition, the sensitivity of cancer cells to apoptosis *in vitro* may be a predictor of their sensitivity to these drugs *in vivo*. We [3,5,28] and others [4,6,34,42,66] have shown that fisetin has significant pro-apoptotic activities against cancer cells. A structure-activity relationship study of 22 flavonoids showed that at least two hydroxylations in positions 3, 5, and 7 of the A ring were needed to induce apoptosis, whereas hydroxylation in 3' and/or 4' of the B ring enhanced proapoptotic activity [70].

Treatment of human epidermoid carcinoma A431 cells with fisetin resulted in decreased expression of anti-apoptotic proteins and enhanced expression of pro-apoptotic proteins.

Moreover, an increase in cell populations with diminished mitochondrial membrane potential, exhibiting loss of mitochondrial integrity was observed indicating that fisetin induced apoptosis involves mitochondrial disruption. The shift in mitochondrial membrane potential was accompanied by release of cytochrome c and Smac/DIABLO levels resulting in activation of the caspase cascade and cleavage of PARP [6].

Structurally related flavonoids including luteolin, nobiletin, wogonin, baicalein, apigenin, myricetin were studied along with fisetin for their biological activities on the human leukemia cell line, HL-60. It was shown that fisetin was a potent inducer of apoptosis in human promyeloleukemic cells. Fisetin mediated activation of caspase-3 was accompanied by an increase in endonuclease activity [66]. Studies conducted in hepatocellular carcinoma SK-HEP-1 cells showed similar apoptotic activity of fisetin with induction of p53 protein [67].

In HCT-116 human colon cancer cells fisetin induced apoptosis was associated with suppressed antiapoptotic Bcl-xL and Bcl-2 and increased proapoptotic Bak and Bim protein levels. Activation of p53 contributed to mitochondrial translocation of Bax via a transcription-independent pathway, thought to be responsible, at least partially, for the apoptosis observed in fisetin treated cells. Additionally, fisetin caused increased protein expression of signaling molecules involved in death receptor (DR) signaling including Fas ligand, DR5 and TNF-related apoptosis-inducing ligand (TRAIL). Cleavage of caspase-8 and fisetin-mediated release of cytochrome c and Smac/Diablo indicated that fisetin induces apoptosis in HCT-116 cells via the activation of both the extrinsic and intrinsic pathways [42]. The effect of fisetin on mitochondrial enzymes with induction of apoptosis was also observed in benzo(a)pyrene-induced lung cancer animal model. Histopathological studies of lung sections of these mice showed the presence of phaeomorphic cells with dense granules and increased mitochondria while these aberrations were alleviated in fisetin treated mice [68].

Securin, over-expressed in several types of cancer, negatively regulates the transcription and subsequent apoptotic activity of the tumor suppressor p53. Securin depletion was shown to sensitize human colon cancer cells to fisetin-induced apoptosis. Fisetin-induced apoptosis was blocked by non-degradable or wild-type securin, suggesting that securin degradation was important for fisetin induced cytotoxicity. Notably, fisetin inhibited securin expression regardless of p53 status, as knockdown of securin enhanced fisetin-induced apoptosis in p53-null HCT116 cells [69]. A small molecule library that screened for candidates that could prevent the binding of the viral oncoprotein E6 to FADD and caspase-8 identified fisetin as a potent inhibitor of HPV16-E6-caspase-8 interaction suggesting the potential of fisetin to induce apoptosis of infected cells [71].

4.6.2. Signaling molecules involved in fisetin induced apoptosis in cancer

cells—Apoptosis by fisetin involves modulation of several signaling pathways. Ying et al. [38] demonstrated that fisetin induced apoptosis in human cervical cancer HeLa cells through ERK1/2-mediated activation of caspase-8 pathway. Treatment of HeLa cells with fisetin induced sustained phosphorylation of ERK1/2, and pharmacological inhibition of ERK1/2 or transfection with a mutant ERK1/2 expression vector significantly abolished

fisetin-induced apoptosis [38]. Fisetin treatment resulted in dose-dependent inhibition of pancreatic cancer cell growth and proliferation with concomitant induction of apoptosis. We showed that fisetin induced apoptosis and inhibited invasion of chemoresistant pancreatic cancer AsPC-1 cells through suppression of DR3-mediated NF- κ B activation [72]. Of more than 20 genes modulated at transcription level in cDNA array studies, maximum decrease was observed in DR3 expression paralleled with an increase in the expression of I κ B α , an NF- κ B inhibitor. Consistent with these findings, knockdown of DR3 or blocking of DR3 receptor with an extracellular domain blocking antibody significantly augmented fisetin induced changes in cell proliferation, invasion and apoptosis associated with decrease in NF- κ B activity [72].

Sung et al. [73] investigated the effect of fisetin on activation of NF- κ B pathway by various carcinogens and inflammatory stimuli. Among the nine different flavones examined, fisetin was found to be the most potent in suppressing tumor necrosis factor (TNF)-induced NF- κ B activation. Inhibition of NF- κ B activation by fisetin subsequent to reduction in degradation of I κ B α and nuclear translocation of p65 was found to be mediated through modulation of kinases including RIP, TAK1 and IKK. This correlated with reduced expression of NF- κ B-regulated gene products including survivin, IAP1, IAP2, XIAP, Bcl-2, Bcl-xL and TRAF1, all known to inhibit apoptosis. In accordance, potentiation of apoptosis induced by doxorubicin, and cisplatin was observed [73]. Additional evidence of its inhibitory effect on the NF- κ B signal transduction pathway came from a study in nasopharyngeal carcinoma cells where fisetin interfered with targets of NF- κ B activated by Epstein-Barr virus encoded LMP1 protein [74].

Fisetin-induced apoptosis in cyclooxygenase (COX)-2-overexpressing HT29 human colon cancer cells was accompanied with inhibition of epidermal growth factor receptor/NF- κ B-signaling pathways [43]. Fisetin down-regulated COX-2 and reduced the secretion of prostaglandin E2 without affecting COX-1 protein expression. Additionally, treatment of cells with fisetin inhibited Wnt signaling activity through suppression of β -catenin and T cell factor 4 and decreased the expression of target genes such as cyclin D1 and MMP-7 [43].

In human bladder cancer cells fisetin-induced apoptosis was mediated via modulation of two related pathways: upregulation of p53 and downregulation of NF- κ B activity, resulting in a change in the ratio of pro- and anti-apoptotic proteins. Fisetin increased the expression of Bax and Bak but decreased the levels of Bcl-2 and Bcl-xL and subsequently triggered the mitochondrial apoptotic pathway [75]. Similar effect was observed with fisetin treatment of an autochthonous rat model where bladder cancer was induced by administration of intravesical N-methyl-N-nitrosourea to the animals. Fisetin significantly reduced the incidence of bladder tumors by activating p53 and suppressing NF- κ B signaling with modulation of NF- κ B target genes involved in cell proliferation and apoptosis [76].

An alternative mechanism identified for fisetin induced apoptosis was through modulation of transcription factor heat shock factor 1 (HSF1) involved in regulation of heat shock proteins (HSPs) expression, crucial in enhancing survival of cancer cells exposed to stress [4]. Fisetin was identified as a potent HSF1 inhibitor in a cell-based screening library of natural

compound. It was shown that the induction of HSF1 target proteins, such as HSP70, HSP27 and BAG3 were inhibited in HCT-116 cells exposed to heat shock at 43°C for 1h in the presence of fisetin [4]. It was demonstrated that fisetin inhibited HSF1 activity by blocking the binding of HSF1 to the *hsp70* promoter. Importantly, fisetin decreased the expression of anti-apoptotic proteins Bcl-2, Bcl-xL and Mcl-1 through down-regulation of their chaperones, HSP70 and BAG3 indicating alternate mechanisms involved in the regulation of apoptotic machinery [4].

4.6.3. Role of reactive oxygen species (ROS) in fisetin induced apoptosis—

Fisetin induced apoptosis in the human non-small cell lung cancer cell line, NCI-H460, as evidenced by apoptotic body formation, DNA fragmentation, an increase in the number of sub-G1 phase cells, mitochondrial membrane depolarization and activation of caspase-9 and caspase-3. This was associated with production of intracellular ROS [77]. Also, in human hepatic Huh-7 cells fisetin induced apoptosis associated with downregulation of BIRC8 and Bcl2L2 was accompanied with intracellular ROS accumulation [78]. Jang et al. [79] showed that fisetin induced apoptosis in multiple myeloma was ROS-dependent and mediated via activation of AMP activated protein kinase (AMPK) pathway. Fisetin induced AMPK signaling was accompanied by activation of its substrate acetyl-CoA carboxylase, along with decreased phosphorylation of Akt and mTOR.

In contrast, our studies in 2-D melanoma cultures showed that fisetin induced cytotoxicity is independent of AMPK activation [5]. Furthermore, fisetin treatment to melanoma cells resulted in inhibition of ROS generation at all-time points studied, from 30 min post treatment to 24 h, signifying that fisetin induced apoptosis is not mediated through ROS generation. Interestingly, a marked increase in nitric oxide (NO) generation was evident with fisetin treatment particularly at extended time points [5]. In agreement with our studies, Ash et al. [80] described NO as a key molecule in fisetin induced cytotoxicity and showed that fisetin induced apoptosis of leukemia cells through generation of NO and elevated Ca^{2+} activating the caspase dependent apoptotic pathways. Fisetin was shown to inhibit the mTORC1 pathway and its downstream components including p70S6K, eIF4B and eEF2K. Interestingly inhibition of NO restored phosphorylation of downstream effectors of mTORC1 and rescued cells from death. In addition abrogation of Ca^{2+} influx reduced caspase activation and exerted a protective effect on fisetin treated cells. The increase in NO was independent of Ca^{2+} levels suggesting that these two phenomena may be mutually exclusive [80]. Others have also noted a similar decrease in ROS with fisetin treatment. Fisetin-induced apoptosis in human promyeloleukemic cells mediated through activation of Ca^{2+} -dependent endonuclease was associated with decrease ROS levels [66].

Ionizing radiation induces cellular oxidative stress through generation of ROS resulting in cell damage and cell death. Fisetin was shown to reduce the levels of intracellular ROS generated by γ -irradiation thereby protecting cells against radiation-induced membrane lipid peroxidation, DNA damage, and protein carbonylation [81]. Remarkably, in a recent study, the known antioxidant N-acetyl-L-cysteine (NAC) was found to enhance fisetin-induced cytotoxicity via induction of ROS-independent apoptosis in human colon cancer cells [40]. However, taking into account the recently identified role of NAC as an antagonist of

proteasome inhibitors, data interpretation might not be straightforward in studies where NAC is utilized as an antioxidant to validate ROS involvement in fisetin-induced cell death.

4.6.4. Fisetin and autophagy in cancer cells—Autophagy, a highly conserved cytoprotective process serves to alleviate various types of stress in the cell. The crosstalk between apoptosis and autophagy is complex, and the mechanisms are still poorly understood. However, this crosstalk may decide cellular fate so that under certain circumstances autophagy can promote cell survival and prevent apoptosis whilst in other cases autophagy may conclude with cellular death with or without apoptosis [82]. Only a handful of studies have looked at the occurrence of autophagy with fisetin treatment and the results are conflicting. We showed earlier that fisetin treatment to PC-3 prostate cancer cells resulted in induction of autophagic-programmed cell death associated with inhibition of mTOR pathway [28]. Interestingly, in another study, fisetin was shown to target caspase-3-deficient MCF-7 breast cancer cells by induction of caspase-7-associated apoptosis and inhibition of autophagy. Treatment of cells with fisetin along with autophagy inhibitors significantly increased the percentage of cells undergoing apoptosis indicating that inhibition of autophagy readily contributes to the growth suppressive effect of fisetin [35]. Using melanoma cells as a prototype we showed that apoptosis is the primary mechanism through which fisetin inhibits melanoma cell growth and that activation of both extrinsic and intrinsic pathways contributes to fisetin induced cytotoxicity. Induction of ER stress upon fisetin treatment, evident as early as 6 h, and associated with up-regulation of IRE1 α , XBP1s, ATF4 and GRP78, was followed by autophagy which was not sustained [5]. This transient autophagic response observed in fisetin-treated melanoma cells was speculated to likely be a defense mechanism by cells against fisetin-induced stress. As is clear from the above citations, in-depth studies in diverse models are needed to analyze the role of autophagy in fisetin-induced cytotoxicity.

4.6.5. Fisetin increases the sensitivity of cells to apoptosis—It was surmised that a combination of compounds that lead to optimal blockade of critical signaling pathways involved in cell proliferation survival and tumorigenesis may provide an effective strategy for the prevention and treatment of cancer. In this context, it was shown that melatonin, a hormone with diverse physiological functions significantly enhanced the anti-tumor activity of fisetin [83]. The combinatorial treatment was much more effective in suppression of cell viability and migration, and induction of apoptosis compared to fisetin alone. It was demonstrated that melatonin potentiated the effect of fisetin in melanoma cells by activating cytochrome c-dependent apoptosis and inhibiting COX-2/iNOS and NF- κ B/p300 signaling pathways [83]. Combination of fisetin and the BRAF inhibitor sorafenib was found to be extremely effective in inhibiting the growth of BRAF-mutated human melanoma cells resulting in enhanced apoptosis, reflected by cleavage of caspase-3 and PARP, increased expression of Bax and Bak, and inhibition of Bcl-2 and Mcl-1 [84]. In addition, synergistic effect of fisetin and sorafenib was observed in human cervical cancer HeLa cells, where *in vitro* and *in vivo* studies revealed that the combination was clearly superior to sorafenib treatment alone. The study identified that the increase in apoptotic potential was mediated through the DR5-dependent activation of caspase-8/caspase-3 signaling pathways [85].

Similarly, fisetin in combination with hesperetin induced apoptosis and cell cycle arrest in chronic myeloid leukemia cells accompanied by modulation of cellular signaling [86]. Gene profiling analysis revealed some important signaling pathways including JAK/STAT pathway, KIT receptor signaling, and growth hormone receptor signaling that were altered upon fisetin and hesperetin combinatorial regimen [86]. Fisetin was shown to synergize with Casodex in inducing apoptosis in LNCaP cells [27].

In another study, HSP90 inhibitors geldanamycin and radicicol enhanced fisetin-induced cytotoxicity in human colon cancer cells via activation of the mitochondria-dependent caspase-3 cascade and accelerated degradation of p53 protein. However the mechanism(s) involved in HSP90 inhibitors-regulated p53-chaperon interactions in the context of fisetin treatment is not well understood [41]. Pretreatment with fisetin enhanced the radio-sensitivity of p53 mutant HT-29 cancer cells, prolonged radiation-induced G2-M arrest, and augmented radiation-induced caspase-dependent apoptosis [87]. This was associated with increased phosphorylation of p38 MAPK, and dephosphorylation of Akt and ERK1/2 [87]. Use of chemotherapeutic drug cisplatin is limited because of its toxicity. Several studies have evaluated the potential of fisetin in enhancing cisplatin-induced cytotoxicity in various cancer models [88,89]. Addition of fisetin to cisplatin enhanced its apoptotic effect several folds, in human embryonal carcinoma NT2/D1 cells. Findings of the study suggested that the combination resulted in activation of mitochondrial and cell death receptor pathways, at significantly lower doses of cisplatin. These findings were validated in a NT2/D1 mouse xenograft model, where again combination therapy was more effective than single regimens in reducing tumor size [88]. In a more recent study in cisplatin-resistant A549 lung cancer cells, fisetin was shown to reverse acquired resistance and increase sensitivity to cisplatin, possibly by inhibiting aberrant activation of MAPK signaling [89].

4.6.6. Protective effect of fisetin against apoptosis—Studies were undertaken to determine whether a cytoprotective dose range of flavonoids could be differentiated from a cytotoxic dose range. Seven structurally related flavonoids were tested for their ability to protect H4IIE rat hepatoma cells against H₂O₂-induced damage and/or to induce cellular damage [88]. Experiments in cell-free systems showed that the flavonoids are potent antioxidants however, their pharmacologic activity correlated with cellular uptake rather than their *in vitro* antioxidant potential. For quercetin and fisetin, which were readily taken up into the cells, protective effect against H₂O₂-induced cytotoxicity, DNA strand breaks, and apoptosis was detected at concentrations as low as 10–25 µM. Conversely, DNA strand breaks, oligonucleosomal DNA fragmentation, and caspase activation was observed at concentrations between 50–250 µM. The data suggested that cytoprotective concentrations of flavonoids are lower by a factor of 5–10 than their DNA-damaging and pro-apoptotic concentrations. Moreover, low concentrations of flavonoids are typically protective whereas high concentrations cause DNA damage and apoptosis [88]. Co-exposure of osteoblast-like MC3T3-E1 and hippocampal HT22 cell lines to fluoride and dexamethasone resulted in a decrease in cell viability, induction of apoptosis and increased generation of ROS and NO. It was demonstrated that fisetin treatment exerted a protective effect and prevented fluoride- and dexamethasone-induced cytotoxicity in osteoblast and hippocampal cells [89].

Collectively these findings support an important role of fisetin in the regulation of apoptotic machinery which can be exploited for destroying cancer cells. However, a therapeutic window for its diverse functions exists which needs to be established.

5. Future perspectives

Because of their wide distribution in the diet, flavonoids are presumed to be extremely safe and associated with little or no toxicity. This aspect assumes significance since many drugs in clinical use are associated with severe side effects. The cancer protective effects of flavonoids such as fisetin have been attributed to a wide variety of mechanisms, including modulating enzyme activities and inhibiting oncogenic pathways. Interest in the anti-cancer activity of fisetin was sparked when it was discovered that fisetin along with other flavonoids modulated activity of enzymes largely responsible for the detoxification of carcinogens. Subsequent studies have suggested that fisetin has both therapeutic and cancer preventive properties. While most of the information about the biological activity of fisetin is based on *in vitro* cell culture based studies there is a need to undertake extensive *in vivo* preclinical studies in relevant animal models. Data from preclinical studies will be crucial in assessing the potential use of fisetin before undertaking any clinical trials.

It is interesting to note that many studies have observed the enhanced efficacy of cancer therapeutic compounds when used in combination with fisetin. Because fisetin binds to multiple targets its enhanced combinatorial effects with conventional therapeutic drugs has been observed to be clinically relevant. Fisetin inhibits both mTOR complexes and its combination with other rapamycin analogues seems plausible. Fisetin could be used in combination with taxols against advanced and resistant forms of cancers. Based on the available literature it remains unexplored why and how fisetin enhances the chemotherapeutic effects of conventional drugs. The existence of a possible chemical synergy when using a combination needs to be explored in detail in terms of the mechanism and clinical relevance. A potential advantage of using a combination is that it could help reduce the effective dose of the therapeutic drug and any associated side effects. Further, studies are needed to understand how the combination works and what molecular pathways are affected with the combination.

Fisetin has been shown to physically interact with multiple signaling molecules. Our own observations suggest its interaction with the mTOR, p70S6K kinase and α -tubulin molecule. While docking studies reveal that fisetin binds to mTOR on three sites located on residues from helices $\alpha 2$ and $\alpha 3$, it was observed that fisetin can bind to mTOR with a different alignment into the binding site and still retain favorable binding energy. In order to ensure a tight fit and enhance binding, modification of the parent molecule could yield better results. Synthesis of fisetin analogues need to be earnestly considered and designed based on existing data on its molecular interactions. Identifying fisetin derivatives with significantly enhanced activity while preserving its biologic activities will be challenging but equally rewarding at the same time. A lingering concern with the use of diet based agents is poor absorption and bioavailability. Issues related to solubility and bioavailability remain a challenge in the ultimate aim of developing it for human use. The pharmacokinetic profile

needs to be thoroughly investigated and will be essential in determining the potential use in the clinic.

Conclusions

Fisetin belongs to a group of flavonoids that have been described as health-promoting, disease-modifying and cancer preventive agents. As a small molecule fisetin has shown activity in many biological assays suggesting that it could potentially be useful against many disease conditions. Essentially, fisetin interferes with many cancer-related pathways (Figure 2) and inhibits cancer by promoting apoptosis and modulating autophagic cell death. By physically interacting with mTOR and p70S6K molecules and disrupting Wnt/ β -catenin signaling fisetin inhibits a host of cell survival pathways. However, detailed understanding of its mechanisms of action could enhance its use and effectiveness.

Drugs that affect microtubule dynamics are among the most effective anticancer agents in routine clinical use. Although the vast majority of known microtubule-stabilizing agents are structurally complex, our finding of fisetin, a small molecule, as a microtubule stabilizing agent being far superior to paclitaxel is both novel and exciting. Combination therapy at much lower doses than the doses already used are needed that will be nontoxic, yet effective in suppressing microtubule dynamics. We suggest that fisetin be explored further for the treatment of cancers, alone or as an adjuvant with other chemotherapy drug. As our understanding of the mechanism(s) of action of fisetin increases, we can exploit these to design strategies that can improve the efficacy of fisetin for the treatment of cancer.

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Figure 1. Fisetin physically interacts with p70S6K

The structure of a domain-swapped dimer of p70S6K (PDB code: 3a60) with bound fisetin (adapted from Syed et al. [31]). Fisetin is shown in green while the protein is represented by cartoon. The figure shows fisetin bound to the ATP binding pocket located on the hydrophobic cleft between the N- and C-terminal domains. Enlarged view of the active site demonstrates disposition and hydrogen bonding interactions (in yellow) between the catechol moiety of fisetin and residues Gly100 and Gly98 involved in molecular recognition.

Figure 2. Fisetin inhibits multiple cellular targets

By binding to and interacting with several molecular targets fisetin disrupts a wide variety of cell functions. Fisetin disrupts Wnt signaling and results in cell cycle arrest. It inhibits the Y-Box 1 binding protein and interferes with epithelial to mesenchymal transition thus preventing the invasion and migration of cancer cells. By physically interacting with the mTOR molecule, fisetin inhibits several downstream signaling explaining its inhibitory effect on cellular growth and proliferation. Fisetin binds to and disrupts microtubule dynamics and as a microtubule stabilizing agent is superior to paclitaxel.

Table 1

Pharmacokinetic parameters of free, nanoemulsion and liposomal fisetin in mice.

Type of fisetin	Dose (mg/kg)	Route of administration	C _{max} (µg/ml)	T _{1/2} (h)	AUC _{0→t} (µgh/ml)	MRT (h)	MAT (h)	Reference
Free fisetin	13	Intravenous	6.0	0.61	1.12	0.97	-	[15]
Fisetin nano-emulsion	13	Intravenous	5.3	0.65	1.13	0.99	-	[15]
Free fisetin	223	Intraperitoneal	2.53	4.19	4.07	6.95	5.98	[15]
Fisetin nano-emulsion	112.5	Intraperitoneal	22.96	3.07	48.53	2.96	1.97	[15]
Liposomal fisetin	13	Intravenous	10.0	3.8	1.84	1.04	-	[14]
Liposomal fisetin	21	Intraperitoneal	6.75	55.4	10.06	1.93	1.48	[14]

T_{1/2}-Terminal half-life; MRT-Mean residence time; MAT-Mean absorption time