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Fisetin inhibits human melanoma cell growth through direct binding to p70S6K and mTOR: findings from 3-D melanoma skin equivalents and computational modeling

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Abstract

The incidence of melanoma continues to rise. In spite of treatment advances, the prognosis remains grim once the disease has metastasized, emphasizing the need to explore additional therapeutic strategies. One such approach is through the use of mechanism-based dietary intervention. We previously showed that the flavonoid fisetin inhibits melanoma cell proliferation, *in vitro* and *in vivo*. Here, we studied fisetin-mediated regulation of kinases involved in melanoma growth and progression. Time-course analysis in 3-D melanoma constructs that transitioned from radial to vertical growth showed that fisetin treatment resulted in significant decrease in melanocytic lesions in contrast to untreated controls that showed large tumor nests and invading disseminated cells. Further studies in melanoma cultures and mouse xenografts showed that fisetin-mediated growth inhibition was associated with dephosphorylation of AKT, mTOR and p70S6K proteins. *In silico* modeling indicated direct interaction of fisetin with mTOR and p70S6K with favorable free energy values. These findings were validated by cell-free competition assays that established binding of fisetin to p70S6K and mTOR while little affinity was detected with AKT. Kinase activity studies reflected similar trend with % inhibition observed for p70S6K and mTOR at lower doses than AKT. Our studies characterized, for the first time, the differential interactions of any botanical agent with kinases involved in melanoma growth and demonstrate that fisetin inhibits mTOR and p70S6K through direct binding while the observed inhibitory effect of fisetin on AKT is mediated indirectly, through targeting interrelated pathways.

Keywords

Fisetin; AKT; mTOR; p70S6K; melanoma

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Introduction

Cutaneous melanoma, a tumor derived from epidermal melanocytes, accounts for more than 50% of all skin cancer-related deaths [1]. Most primary melanomas arise in the epidermis as horizontal plaque-like lesions, termed the radial growth phase (RGP) which progress to the invasive vertical growth phase (VGP) ultimately leading to distant metastasis. The molecular changes involved in melanoma development, include deregulated activity of kinases and transcription factors [2]. In this context, transition from RGP to VGP has been associated with activation of the serine-threonine kinase, AKT [3]. AKT, located in the cytoplasm, upon activation of PI3K is recruited to the cell membrane where it is phosphorylated at T³⁰⁸ residue by membrane-localized kinase, PDK-1 [4]. The mammalian target of Rapamycin (mTOR), an important effector of PI3K/AKT pathway phosphorylates AKT at the S⁴⁷³ residue. Phosphorylation at both residues is required to fully activate the catalytic activity of AKT [4]. Studies in melanoma models indicate that AKT acts as a molecular switch that increases angiogenesis and generation of superoxide, fostering aggressive behavior in melanoma tumors [5]. In addition, AKT decreases transcription of the E-cadherin adhesion protein permitting detachment of adjacent cells [5, 6].

Recent data indicate that activation of the mTOR pathway is correlated with poor prognosis in melanoma patients [7]. The mTOR kinase, with a key role in cell proliferation, resides in two different complexes; mTORC1 and mTORC2, comprising mTOR, mLST8/GβL and Deptor [8]. The presence of Raptor and PRAS40 defines mTORC1, whereas the presence of Rictor, mSin1 and Protor1/2 defines mTORC2 [8]. The mTORC1 sensitive to rapamycin, integrates inputs from diverse environmental cues to regulate cell growth and proliferation [8]. The ribosomal protein S6 kinase (S6K) and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) are well- characterized substrates of mTORC1. S6K consists of a 70-kDa cytoplasmic isoform (p70S6K) and an 85-kDa nuclear isoform (p85S6K) both of which phosphorylate the S6 protein and mediate the translation of polypyrimidine tract mRNA, including transcripts of ribosomal proteins and translational elongation factors [9]. mTORC1 mediated phosphorylation of 4E-BP1 initiates cap-dependent translation by eukaryotic translation initiation factor 4E (eIF4E) [10]. Thus mTORC1 signals along parallel pathways to coordinately promote protein synthesis, cell proliferation and cell cycle progression [8]. The function of mTORC2 is not entirely understood however, it is known that mTORC2 phosphorylates AKT, SGK1 and PKCα kinases on their hydrophobic motif sites and has a role in cell proliferation, survival and metabolism [8].

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. Moreover, there is a need to target multiple pathways that regulate the various feedback loops reportedly involved in melanoma progression. Fisetin, a tetrahydroxyflavone present in fruits and vegetables is being investigated for its antiproliferative activity against several human cancers [11]. We previously showed that fisetin inhibits melanoma cell growth *in vitro* and in melanoma xenograft models [12]. Here, we examined the effect of fisetin on signaling molecules involved in melanoma progression employing a 3-D human skin equivalent melanoma model that reproduces the transition of melanoma from RGP to VGP. Specifically, we examined the mechanisms through which

fisetin exerts its inhibitory effect on AKT, mTOR and p70S6K and established the differential binding affinity of fisetin to these kinases, using docking studies and competition assays.

Materials and methods

Materials

Fisetin was purchased from Sigma (St. Louis, MO). All antibodies were obtained from Cell Signaling Technology (Danvers, MA). The malignant melanoma human tissue array with normal skin control tissue (Cat # T382) was purchased from US Biomax Inc (Rockville, MD).

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. Cells (70% confluent) were treated with fisetin dissolved in DMSO (10–60μM) for specified time points and harvested for further studies.

Melanoma skin model

The full-thickness melanoma skin model obtained from MatTek Corporation (Ashland, MA) consists of A375 melanoma cells, epidermal keratinocytes and dermal fibroblasts cultured to form a multilayered, highly differentiated human epidermis. At different stages of culture, the tissue exhibits RGP, VGP, or metastatic melanoma phenotype. Throughout the experiment, the tissue was maintained at 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 (40 and 80μM) after which samples were harvested at 4, 8, 12 and 16 days for further studies.

Cell viability

Cell viability was determined by 3-(4,5 dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay as described previously [12].

Cell cycle analysis

451Lu treated with/without fisetin for 48 h were processed for labeling with fluorescein-tagged dUTP nucleotide and propidium iodide using the APO-DIRECT™ kit (Phoenix Flow Systems, CA) and analyzed using Modfit software.

Preparation of cell lysate

Whole cell lysates with/without fisetin treatment were prepared for western blot analysis as described previously [12]. Densitometric analyses of protein expression, normalized to β-actin, was performed using the Image J software.

Immunoprecipitation

Briefly, specific antibody was added to 100µg of cell lysates after pre-clearing with protein A/G-agarose beads. After overnight incubation, protein A/G agarose beads were added and left for 3 hours. After centrifugation and several washes, the pellet was resuspended with 2X sample buffer and electrophoresed on a SDS-PAGE gel.

Immunohistochemistry

Briefly, sections from melanoma constructs/xenografted mouse tissues, fixed in 10% formalin were deparaffinized and rehydrated in xylene and a graded series of ethanol and blocked in 2% goat serum/PBS for 30 minutes. After incubation with specific primary antibody overnight and HRP-conjugated secondary antibody for 2 h, slides were counterstained with H&E and immunoreactive complexes were detected using 3,3'-diaminobenzidine (Dako Corp., CA). Sections were visualized on Olympus CKX41 microscope and images captured with a camera attached to computer. Figures were composed using ADOBE PHOTOSHOP 7.0 (Adobe Systems, CA).

Computational methods

Ligand docking studies were performed using Molecular Operating Environment software package (MOE, version 2009.10, Chemical Computing Group Inc., Montreal, Canada). Three-dimensional structures of mTORC1, AKT-[(pleckstrin homology (PH) domain)], and p70S6K1 used for docking experiments were downloaded from the PDB Data Bank (<http://www.rcsb.org/> - PDB codes: 2npu [13], 1unq [14], 3a60 [15]). Before docking, the protein structures were properly protonated using the Protonate 3D option, and were geometrically optimized and minimized by employing MMFF94x force field with a generalized Born solvation model, and RMSD gradient $0.05 \text{ kcal mol}^{-1} \text{ \AA}^{-1}$. The partial charges were automatically calculated and only the water molecules in the proximity of the binding site were retained. The ligand structures [i.e., fisetin, inositol-(1,3,4,5)-tetrakisphosphate (IP4) and staurosporine] were built in MOE and minimized before the docking, using the MMFF94x force field, with the steepest descent algorithms until a RMSD gradient of $0.05 \text{ kcal mol}^{-1} \text{ \AA}^{-1}$ was reached. The binding sites were broadly defined based on residue proximity to the binding conformation of each ligand within the respective target protein. Rigid receptor-flexible ligand docking calculations were performed using the docking simulation feature MOE-dock by setting grid sizes that included the entire macromolecules. The triangle matcher was used as placement method to generate docking poses [16], and the London free energy values scoring function that estimates the free energy of binding (kcal/mol) was used to rank hit candidates, after a force field based refinement and rescoring. Only the best scored poses generated for each ligand in all the docking experiments were retained and examined with MOE.

Kinase binding assays

The KINOMEscan (San Diego, CA) screening platform was employed to quantitatively measure interactions between fisetin and AKT, mTOR and p70S6K. A ligand with a known active site for the kinase is immobilized on a solid support. The binding constants (K_d s) for test compound-kinase interactions are calculated by measuring the amount of kinase

captured on the solid support as a function of the test compound concentration. Binding reactions were assembled by combining kinases, liganded affinity beads, and test compound in 1x binding buffer (20% Seablock, 0.17x PBS, 0.05% Tween 20, 6 mM DTT). All reactions were performed in polystyrene 96-well plates in a final volume of 0.135 ml. The assay plates were incubated at room temperature with shaking for 1 h and the affinity beads were washed with wash buffer (1x PBS, 0.05% Tween 20). The beads were then re-suspended in elution buffer (1x PBS, 0.05% Tween 20, 0.5 μ M non-biotinylated affinity ligand) and incubated at room temperature with shaking for 30 minutes. The kinase concentration in the eluates was measured by qPCR. Kds were calculated with a standard dose-response curve using the Hill equation: $\text{Response} = \text{Background} + [\text{Signal} - \text{Background}] / [1 + (\text{Kd}^{\text{Hill Slope}} / \text{Dose}^{\text{Hill Slope}})]$. The Hill Slope was set to -1. Curves were fitted using a non-linear least square fit with the Levenberg-Marquardt algorithm.

Kinase activity assays

In vitro profiling of mTOR and p70S6K and AKT kinases was performed at Reaction Biology Corporation (Malvern, PA). Briefly, the specific kinase/substrate pair along with required cofactors were prepared in reaction buffer [20 mM Hepes pH 7.5, 10 mM MgCl₂, 1 mM EGTA, 0.02% Brij35, 0.02 mg/ml BSA, 0.1 mM Na₃VO₄, 2 mM DTT, 1% DMSO]. The compounds (fisetin/rapamycin/stauropsporine) dissolved in DMSO were delivered into the kinase reaction mixture, after which ³²P-ATP (specific activity 10 nCi/nl) was added to initiate the reaction. After incubation for 2 h at room temperature, reactions were spotted onto P81 ion exchange filter paper (Whatman Inc., Piscataway, NJ). Unbound phosphate was removed by extensive washing of filters in 0.75% phosphoric acid. After subtraction of background derived from control reactions containing inactive enzyme, kinase activity data was expressed as the percent remaining kinase activity in test samples compared to vehicle (DMSO) reactions. IC₅₀ values and curve fits were obtained using Prism GraphPad software.

Statistical analysis

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

Results

Fisetin inhibits A375 melanoma growth in monolayer cell cultures

Preliminary studies were performed in monolayer cell cultures to ascertain the effect of fisetin on melanoma cells. As assessed by MTT assay, 24 h treatment of A375 melanoma cells with fisetin (20–80 μ M) inhibited proliferation and decreased cell viability by 32, 47, 51 and 52%. The decrease in cell viability was more significant at 48 h where 50% inhibition was observed at ~20 μ M (Fig.1A). The IC₅₀ of fisetin was calculated to be 38.1 and 20.3 μ M at 24 and 48 h respectively. Next, flowcytometric analysis was used to establish the cell cycle profile of fisetin-treated melanoma cells. A375 melanoma cells treated with fisetin for 48 h revealed 1.3, 4.3, 9.3 and 11.3 % cells in the G₂ phase at 0, 10, 20 and 40 μ M respectively, signifying that fisetin induced cell growth inhibition, through arresting cells in the G₂ phase of the cell cycle (Fig.1B,C).

Fisetin inhibits melanoma progression in a 3-D melanoma skin model

We then employed a 3-D full thickness melanoma skin model (MatTek Inc.) comprised of A375 melanoma cells, epidermal keratinocytes and dermal fibroblasts, and proceeded to examine the effect of fisetin on melanoma progression. The concentration of fisetin required for the treatment of 3-D constructs was determined using the formula: $(IC_{50} \text{ monolayer; } 24 \text{ h})/2^*3$. Two concentrations, 40 and 80 μM of fisetin were used to treat melanoma reconstructs over a period of 16 days (Table 1). The constructs were harvested every four days. The first time point at day 4 represented the transition of melanoma growth from RGP to VGP, which then progressed to the metastatic phenotype at day 16 (Fig.1D). H&E-stained images of cross-sections of skin constructs treated with fisetin, at days 12 and 16 showed a dramatic decrease in melanoma cells versus the control skin (Fig.1D). The untreated skin contained nests of tumor cells and many invading disseminated cells in contrast to fisetin-treated skin which had significantly less melanocytic lesions. At day 16, melanocytic lesions were barely detectable in the fisetin-treated skin tissue (Fig.1D & E). More importantly, fisetin caused no detectable damage to the keratinocytes, fibroblasts, or skin morphology present in this model. The effect of fisetin treatment on melanocytic lesion development was quantified overtime, through serial measurement of melanoma constructs that showed a significant ($p<0.05$) decrease in melanoma tumor nests in fisetin-treated constructs compared with controls (Fig. 1E).

Fisetin mediated melanoma growth inhibition is associated with downregulation of mTOR

mTOR has been implicated in enhanced growth and proliferation of melanoma cells [17]. We evaluated the expression of phosphorylated mTOR (p-mTOR) (S^{2448}) in a melanoma tissue microarray that contained tissues from patients diagnosed with malignant melanoma (Table 2). Increased expression of p-mTOR was observed in more than half of the melanoma cases studied, whereas normal skin showed only modest staining of the protein (Fig.2A). Next, we examined the status of phosphorylated mTOR in the 3-D melanoma constructs, at different stages of melanoma development. Immunohistochemical analysis of untreated melanoma constructs showed increased expression of p-mTOR which correlated with melanoma progression (Fig.2B). In contrast, fisetin-treated melanoma constructs showed diminished staining of p- mTOR protein versus the untreated controls. This difference was significantly pronounced at day 16, where melanoma cells showed remarkably less staining for p-mTOR expression. For further validation, we compared p-mTOR expression in archival tissue samples of fisetin-treated 451Lu melanoma xenografts. A similar trend was observed on immunohistochemical analysis where decreased phosphorylation of mTOR protein was observed in fisetin-treated tumor tissues (Fig.2C)

AKT inhibition *in vitro* and *in vivo* with fisetin treatment

Since AKT is believed to contribute to mTOR activation directly or indirectly, we examined the effect of fisetin on AKT activation. A significant decrease in phosphorylated AKT (S^{473}) levels was observed in the fisetin-treated melanoma reconstructs (Fig.3A). We reverted to melanoma (A375 and 451Lu) monolayer cultures and examined the status of phosphorylated AKT in fisetin-treated melanoma cell lysates. Western blot analysis of melanoma cell cultures showed a dose-dependent decrease in phosphorylated forms of both mTOR and

AKT with fisetin treatment (Fig.3B & C). Further studies in tumor lysates of fisetin-treated animals showed similar decrease in phosphorylated mTOR and AKT and an increase in TSC2 protein levels (Fig.3D).

Fisetin dephosphorylates p70S6K and represses downstream targets

Karbowniczek et al, using immunostaining in melanoma tissues have reported an association between p70S6K hyperphosphorylation and melanoma progression [17]. We found invasive growth of melanoma cells into the dermis and hypodermis with increased p70S6K phosphorylation in untreated 3-D melanoma constructs (Fig.4A). In contrast, a significant decrease in the phosphorylated protein expression of p70S6K was observed in the fisetin-treated group. These findings were reproduced in A375 (Fig.4B) and 451Lu (data not shown) monolayer cell cultures where fisetin treatment resulted in a dose-dependent decrease in the phosphorylation of p70S6K at Thr³⁸⁹ and its downstream targets, 4E-BP1 at Thr^{37/46} and eIF4B, indicating that fisetin induced growth inhibition through targeting AKT/mTOR signaling and its downstream effectors.

Fisetin inhibits the

Fisetin does not inhibit AKT through direct binding

To examine whether fisetin exerted its inhibitory effect on AKT through physical interaction at the molecular level, a comparative preliminary docking study was performed, using putative allosteric interference with the pleckstrin homology (PH) domain. Fisetin showed different orientation patterns and binding modes on its hypothetical disposition within the binding site of the AKT-PH protein, with respect to that of IP4, used as a reference ligand. Molecular replacement of IP4 with fisetin did not form a stable complex, as evident by much greater free energy (G_b) values, compared with those of IP4, ($G_b = -6.4293$ vs -15.4895 kcal/mol), respectively (Fig.5A).

To rationalize the observed inhibition of AKT by fisetin, we asked whether fisetin exerted its effect through targeting interconnected pathways that feed into AKT. The mTORC2 is known to phosphorylate conserved motifs on AKT. In addition, there exists a complex feedback regulation of PI3K/AKT signaling by mTORC1. AKT both regulates and is regulated by the TSC1-TSC2 complex [18]. Fig.5B shows that fisetin interfered with mTORC signaling through decreasing the functional interaction between mTOR, Raptor and Rictor. Moreover, fisetin increased the total protein levels of TSC1 and TSC2 and stabilized the binding of TSC1 to TSC2 (Fig.3D&Fig.5C). A concomitant decrease in PI3Kp110 catalytic subunit protein expression in fisetin-treated A375 and 451Lu (data not shown) cells suggests another possible mechanism of AKT inhibition (Fig.5D).

Fisetin binds directly to p70S6K and mTOR

We next determined the interaction of fisetin with p70S6K by docking to its active site. The kinase inhibitor staurosporine, with good affinity and inhibitory activity toward p70S6K, docks at its ATP binding pocket situated at the hydrophobic cleft between the N- and C-terminal domains [15]. Comparative docking of fisetin showed a consistent binding mode and tight affinity within the amino acid pocket located on the N-terminal domain that

included the following residues: Gly98, Lys99, Gly100, Gly103, Val105, Lys 123, Leu172, Thr235, Asp236, Phe237 (Fig.6A). In particular, favorable H-bond interactions were observed between the amino acid residues Gly98 and Gly100 and the catecholic groups of fisetin. The other amino acid residues involved in van der Waals and hydrophobic interactions were located near the catalytic site. The estimated G_b values for the best-scoring poses of fisetin (the top ranked pose in each cluster indicates a close binding site) and staurosporine were in the order of -9.12 and -8.94 kcal/mol, indicating good ligand-receptor affinities (Fig.6A).

In accordance with the solved X-ray crystallography data, the structure of mTOR consists of four stacked α helices, where the cyclic macrolide mTOR inhibitor rapamycin binds on the shallow hydrophobic patch represented by the side chains of residues from helices $\alpha 1$ and $\alpha 4$ [13]. Fisetin was found to bind to three sites on mTOR protein located on residues from helices $\alpha 2$ and $\alpha 3$ (Fig.6B). The first conformation places the catecholic moiety in close proximity to the carboxylate group of Glu2082, whereas the α -keto-hydroxyl functional groups of the chromen-4-one ring, and the hydroxyl functionality in position 7 (7-OH) of the chromen-4-one ring system, interact with Glu2059 and Asn2071, respectively. An additional arene-arene stacking between the phenyl and the imidazole ring of His2055 was observed (Fig.6B, *right*). In the second pose the fisetin backbone is involved in stacking with a hydrophobic pocket of the protein (Fig.6B *middle*), also establishing H-bonding with Glu2052. Fisetin also bound to mTOR in an alternate conformation, where the catechol backbone is rotated 90° with respect to the plane of the chromenone, positioning the hydroxyl groups at close proximity to the side chain of Glu2052, while the α -keto-hydroxyl groups interact with Glu2082 (Fig.6B, *left*). Calculated G_b for these conformations ranged from -9.85 to -10.51 kcal/mol (Fig.6B).

Fisetin binds to p70S6K with higher affinity than mTOR

Using the Kinomescan screening platform, ATP site-dependent competition binding assays were performed to quantitatively measure interactions between fisetin and target kinases. The reaction does not require ATP, and the thermodynamic interaction affinities are more accurately measured. For the assay, tagged kinases, expressed as fusions to T7 bacteriophage and immobilized 'bait' ligands were combined with fisetin. Binding of fisetin to the kinases (directly or indirectly) occludes the ATP site, so that fewer protein molecules bind the immobilized ligand on the solid support. The results are read out by quantifying the amount of fusion protein bound to the solid support, by qPCR (Fig.7A,B). The interactions of fisetin with mTOR, p70S6K and AKT were analyzed, providing affinity data in the nanomolar range. A negative control CDC2L1 was used for comparison studies. Remarkably, fisetin exhibited higher affinity for p70S6K (940nM) than for mTOR (11000nM) whereas no affinity was observed for AKT (40000nM) (Fig.7A,C; Table 3), indicating that direct binding of fisetin to the AKT molecule is not the primary mechanism of fisetin-mediated AKT inhibition.

Fisetin mediated inhibition of p70S6K and mTOR kinase activities occurs at a lower dose than AKT

Kinase activity assays were performed to examine whether binding of fisetin to the kinases could be correlated to inhibition of the functional activity. We utilized a radiometric assay from Reaction Biology Corp. that directly measures the kinase catalytic activity toward a specific substrate. In this assay format, the kinase reactions were performed in the presence of ^{32}P - γ -ATP, followed by binding of the final radioisotope labeled products to filters, after which unreacted phosphate was washed away so as not to interfere with the detection of real phosphorylated products. Comparative studies with reference compounds showed that rapamycin had no effect on mTOR kinase activity when it was not in a complex with FKBP12. In contrast, fisetin inhibited mTOR activity ($6.71\text{E-}06\text{ M}$) independent of FKBP12 (Fig.8A,B). However, rapamycin was more effective than fisetin at inhibition of the mTOR-FKBP12 complex ($3.58\text{E-}08\text{M}$ vs $6.05\text{E-}06$) (Fig.8C). The other reference compound staurosporine was a potent inhibitor of both AKT and p70S6K ($5.27\text{E-}10\text{ M}$ and $8.60\text{E-}09\text{ M}$) as compared to fisetin ($1.44\text{E-}05\text{ M}$ and $5.10\text{E-}06\text{ M}$) (Fig.8D,E). Notably, IC_{50} values derived from the activity based assays demonstrated the same trend as the K_d values derived from the binding assays with fisetin showing a lower IC_{50} for p70S6K than mTOR ($5.10\text{E-}06\text{ M}$ and $6.71\text{E-}06\text{ M}$) and a relatively higher IC_{50} for AKT ($1.44\text{E-}05\text{ M}$) (Fig.8A).

Discussion

Identification of signaling pathways regulating melanoma progression and drug resistance has the potential to lead to the development of novel, more effective agents for inhibiting this deadly form of skin cancer. Several naturally occurring compounds are being investigated for their role in the prevention of cancer and may represent the future modality of the treatment [19]. A plethora of evidence indicates that the AKT/mTOR pathway plays a functionally important role in melanoma [4, 5, 17]. In addition, this serves as a convergence point for growth stimuli that contribute to the initiation and maintenance of melanoma growth [7, 17]. Many cancer-driving mutations including those in *PTEN*, *PIK3CA*, and *TSC1/2* lead to the activation of the AKT/mTOR pathway that in turn stimulate p70S6K, implicating these kinases as potential targets for anti-melanoma drugs [20].

Signaling pathways can be studied optimally when cells are spatially organized to display the architectural features seen *in vivo*, which are uncoupled and lost in two-dimensional culture systems. For our *in vitro* studies we used both monolayer and 3-D melanoma reconstructions to establish the effect of fisetin in melanoma cells. The 3-D tissue exhibits RGP, VGP or metastatic melanoma phenotype thereby closely paralleling *in vivo* melanoma development. Our data showed that fisetin-treated constructs did not show progression of melanocytic lesions, evident from a significant decrease in melanoma cells in the hypodermis and dermis. This was associated with concomitant inhibition of phosphorylation of AKT, mTOR and p70S6K proteins, witnessed in *in vitro* studies as well as xenograft tissues treated with fisetin. Previous studies by our group and others have linked suppression of mTOR signaling to fisetin-induced cell death in various cancer cell types [21–23]. Increased caspase-3 staining observed in fisetin-treated constructs with PARP cleavage and activation of intrinsic apoptotic pathway in fisetin-treated monolayer cultures (data not

shown) suggested that fisetin mediated decrease in melanoma growth is associated with decreased melanoma cell viability. Xenograft studies further corroborated our findings where inhibited melanoma growth accompanied dephosphorylation of AKT, mTOR and p70S6K in fisetin-treated samples.

The primary objective of this study was to study the mechanism(s) through which fisetin interacts with AKT, mTOR and p70S6K kinases. Molecular modeling simulations, such as docking studies, represent a powerful approach to understand the mechanism of action of an endogenous ligand or a bioactive molecule and elucidate molecular features responsible for specific ligand-protein interactions [24]. These have been applied with success to analyze features of the active site, including various hydrophobic and hydrophilic molecular fields that can identify areas for ligand docking [24]. Docking results obtained from the inspection of the highly basic binding pocket for phosphoinositide substrates of AKT-PH protein showed both ineffective interactions and much higher G_b values for fisetin with respect to IP4 (Fig.5A). This may be due to a shallow ligand binding pocket, where charge/charge interactions dominate binding, and do not allow interaction between fisetin and the group-binding residues [14]. Interestingly, fisetin has been consistently shown to inhibit AKT phosphorylation and activation in several cancer cell lines [25]. In addition, PI3K, upstream of AKT has been found to be inhibited by fisetin in several study models [11, 25]. Our data show that fisetin modulates known regulators of AKT signaling including PI3K, TSC and mTORC in melanoma cells (Fig.2,3&5). In the absence of direct binding, it is plausible that the effect on AKT is mediated primarily through negative regulation of stimulatory inputs derived from interrelated pathways targeted by fisetin. However, further studies are warranted to delineate the precise mechanism(s).

An important finding of our study is that fisetin inhibits p70S6K through direct binding at its ATP pocket (Fig.6&7). The p70S6K protein structure includes the N-terminal domain (residues 85–172), composed largely of β -sheet, and the C-terminal domain (residues 173–371), which is predominantly α -helical. The p70S6K inhibitor staurosporine is known to bind at the ATP binding pocket situated at the hydrophobic cleft between the N- and C-terminal domain [15]. Analysis of the docking results reveals that both fisetin and staurosporine fit within the active site, albeit in different positions, with some overlapping features (Fig.6A). Fisetin showed a consistent binding mode and was accommodated well on the above-mentioned amino acid residues. In close proximity, the other residues considered important for the activity of p70SK6 were also involved in substrate/ligand binding [15]. Additionally, comparable estimated G_b s values (~ -9 kcal/mol) for fisetin and staurosporine at the docked positions were observed (Fig.6A). Similar docking studies with mTOR (Fig.6B) revealed that fisetin binds to mTOR on three sites located on residues from helices α_2 and α_3 , where the first two are coherent with that previously described by us [26]. Interestingly, it was observed that fisetin can bind to mTOR with a different alignment into the binding site (Fig.6B, *right*), and still retain favorable G_b (within ~ 1 kcal/mol). These data clearly indicated that fisetin favorably interacts with mTOR and p70S6K in contrast to its interactions with AKT.

Relying solely on computed scores is clearly not sufficient, as docking and scoring studies rely on many approximations. The application of these techniques in concert with other

experimental assays is thus essential to extend our understanding of the mechanism(s) of action of the ligand. Quantitation of binding activity using competition assays validated our earlier observations (Fig.7; Table 3). Fisetin, at the doses tested (0–100 μ M) displayed higher affinity to p70S6K than to mTOR, evident from low dissociation constants (K_d 940 versus 11000 nM) in contrast to AKT, where negligible binding was observed.

Protein kinases are phosphoryl transferases that transfer the γ -phosphate of ATP to conserved serine, threonine, or tyrosine residues on specific substrate proteins. The post-translational modification of proteins with phosphate leads to changes in conformation with subsequent modulation of binding, catalysis and recruitment of effector molecules that regulate cellular signaling pathways [27]. We used the radiometric filtration binding assay, in which incorporation of radiolabeled phosphate into the kinase substrate is assessed as a measure of kinase phosphoryl transfer activity. We observed that fisetin was relatively more effective in inhibiting p70S6K/mTOR kinase activity than AKT, reflecting the trend observed with binding assays (Fig.8). Rapamycin and its analogs are known to form tight binary complex with the small immunophilin FKBP12, which then destabilizes mTORC1 and may interfere with the assembly of the mTORC2 [28]. Remarkably, in the comparison studies with rapamycin, the pharmacologic action of fisetin involved FKBP12 independent inhibition of mTOR kinase activity, highlighting the efficacy of fisetin as an mTOR inhibitor (Fig.8B & C).

The binding assays and activity assays are different in nature so the absolute numbers may not be compared. The first approach uses ATP site-dependent competition binding assays in which standard kinase inhibitors are biotinylated and immobilized with streptavidin-coated magnetic beads. A compound with a high binding affinity for the target kinase will compete the tagged kinase away from the bound ligand, which will then be eluted and quantified by qPCR. In the activity assays the reaction is performed in the presence of radiolabeled ATP to evaluate kinase activity. The constructs of the proteins used in the two assays are different, where the binding assays employ phage displayed protein while the activity assays utilize recombinant purified proteins. Also the endpoints are not the same. Binding assay is a probe replacement assay, where the K_d values are affected by the probe selected. Thus, if the probe and fisetin are binding to the same pocket, then it is a direct competition, otherwise, the competition may not be accurate to measure. The activity based assay directly measures the enzyme activity without probe interference and fisetin can bind anywhere to affect protein folding and subsequently the activity. Even with these challenges, our data from both assay systems demonstrate that fisetin is an effective inhibitor of p70S6K and mTOR kinases. The complexity of the signaling observed in melanoma calls for a network biology approach. It is apparent that attacking the network at only one nodal point is unlikely to make the system fail, as there will be other network connections that may allow the cells to circumvent the given signaling pathway under attack. Accordingly, only targeting the network at the most connected points can lead to system failure. Here we show that fisetin targets several signaling molecules crucial to melanoma progression, albeit through different mechanisms of action. Fisetin inhibits mTOR and p70S6K through direct binding while AKT inhibition is mediated presumably through modulation of interconnecting signaling pathways. By integrating the underlying mechanism of growth inhibition, our findings suggest that fisetin can be developed as a potential agent against

melanoma. Ongoing studies in our lab, in transgenic melanoma mouse models that faithfully mimic the genetic and biological evolution of the disease will further demonstrate the potential of fisetin in the prevention and/or treatment of melanoma.

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Fig.1. Fisetin inhibits A375 melanoma cell growth in monolayer and 3-D cell cultures

(A) *Effect of fisetin on A375 melanoma monolayer cultures:* (A) A375 cells were treated with varying doses of fisetin for 24/48 h for MTT assay. Data expressed as % cell viability represent mean±standard errors of three experiments where $P < 0.05$ was considered significant. (B&C) *Effect of fisetin on A375 melanoma cell cycle profile:* A375 cells treated with fisetin (0–40μM:48 h) were stained with PI/FITC using Apo-Direct kit as per vendor's protocol. Following FACS analysis, cellular DNA histograms were analyzed by ModifitLT V3.0. Data are representative of duplicate tests (B). Graphical representation of the cell cycle data representative of duplicate experiments. (D&E) *Effect of fisetin on melanoma growth in 3-D melanoma skin equivalents:* H&E-stained cross-section of skin constructs (10x) taken at four day interval with or without fisetin (80 μM) treatment (D). Fisetin treatment protocol for 3-D melanoma skin equivalent where two doses 40 and 80 μM, were used to treat A375 constructs, on alternate days for a total duration of 16 days (Table 1). The number of tumor nests in control and fisetin-treated group were quantified. At least three fields were selected from four sections obtained for the treated/untreated groups. Data expressed as number of tumor nests represent the mean±standard errors, where * $p < 0.05$ and ** $p < 0.001$ vs control was considered significant (E).

Fig.2. Fisetin mediated melanoma growth inhibition is associated with downregulation of mTOR
 (A) *mTOR* expression in melanoma tissue: Representative photomicrographs (10x) showing staining for p-mTOR in normal skin and melanoma tissues in a human melanoma tissue microarray. The details regarding age, sex and tissue type in the melanoma tissue microarray are provided in Table 2. (B) *Effect of fisetin on mTOR expression in 3-D melanoma model*: Representative photomicrographs (10x) showing p-mTOR expression in A375 melanoma constructs treated with fisetin (80μM), harvested at day 12 and 16 post treatment. (C) *Effect of fisetin on mTOR expression in 451Lu melanoma xenografts*: Representative photomicrographs (10x) for immunohistochemical staining for p-mTOR in tumor tissues from athymic nude mice treated with 1 mg fisetin intraperitoneally. Data represent samples from each group repeated twice with similar results.

Fig.3. AKT inhibition and upregulation of TSC with fisetin treatment

(A) *Effect of fisetin on p-AKT expression in 3-D melanoma model*: Representative photomicrographs (10x) showing p-AKT in A375 melanoma constructs treated with fisetin (80 μ M), harvested at days 12 and 16 post treatment. (B&C) *Effect of fisetin on p-AKT and p-mTOR expression in A375 (B) and 451Lu (C) monolayer cell cultures*: Whole cell lysates of A375 and 451Lu melanoma cells treated with fisetin (20–60 μ M;24 h) were analyzed for expression of various proteins. Equal loading was confirmed by β -actin (left). Data shown are representative of three independent experiments with similar results. Relative density of the bands normalized to β -actin (right). (D) *Effect of fisetin on p-AKT expression in 451Lu melanoma xenografts*: Whole cell lysates of tumor tissue from athymic nude mice, treated with 1 mg fisetin intraperitoneally, were analyzed by western blotting. Equal loading was confirmed by β -actin (left). Data represent samples from each group repeated twice with similar results. Relative density of the bands normalized to β -actin (right).

Fig.4. Fisetin dephosphorylates p70S6K and represses its downstream targets
 (A) Effect of fisetin on p-p70S6K expression in 3-D melanoma model: Representative photomicrographs (10x) showing p-p70S6K in A375 melanoma constructs treated with

fisetin (80 μ M), harvested at days 12 and 16 post treatment. (B) *Effect of fisetin on p70S6K expression and its downstream targets in melanoma monolayer cultures:* Whole cell lysates of A375 cells treated with fisetin (20–60 μ M:24 h) were analyzed for expression of various protein. Equal loading was confirmed by β -actin (*left*). Data shown are representative of three independent experiments with similar results. Relative density of the bands normalized to β -actin (*right*).

Fig.5. Fisetin does not inhibit AKT through direct binding

(A) *In silico docking of fisetin*: Hypothetical disposition of fisetin on the AKT phosphoinositide substrate PH-domain. The protein is represented by cartoon (left) and as molecular surface (right) and amino acid residues and ligands involved in mutual molecular interactions are shown as wireframes and sticks, respectively. (B&C) *Effect of fisetin on mTORC and TSC*: Immunoprecipitation studies of fisetin treated cells (60 μ M:24 h) using mTOR and TSC2 antibodies, probed for Raptor /Rictor (B) and TSC1 (C); bottom panel shows protein expression of input lysates. (D) *Effect of fisetin on PI3K expression in monolayer cell cultures*: Whole cell lysates of A375 melanoma cells treated with fisetin (20–60 μ M:24 h) were analyzed. Equal loading was confirmed by β -actin (*top*). Data shown are representative of three independent experiments with similar results. Relative density of the bands normalized to β -actin (*bottom*).

[B]

Fig.6. Fisetin binds directly to p70S6K and mTOR

(A&B) *In silico docking of fisetin*. Proteins are represented by cartoons/molecular surfaces, and all amino acid residues and ligands involved in mutual molecular interactions are shown as wireframes and sticks, respectively. (A) Proposed binding mode of fisetin within p70S6K staurosporine binding site (*upper panel*). Dashed *blue* arrows denote H-bonds to residue backbone, and arrowheads indicate their direction i.e. donor is at base of the arrow, whereas acceptor is at the head (*lower panel, left*). Amino acid residues (circles) shown as: hydrophobic residues (green interior), polar residues (light purple), basic residues (blue ring), acidic residues (red ring). Differences in solvent accessible surface area for fisetin and receptor residues are plotted as *blue* smud and turquoise halo, respectively. The border of binding pocket is marked by a dashed grey line. The structure of a domain-swapped dimer of p70S6K (PDB code: 3a60) with bound fisetin (*lower panel, right*). The overall topology of p70S6K resembles other AGC kinase folds. The protein includes, for both chains, the N-terminal domain (residues 85–172), composed largely of β -sheet, and the C-terminal domain (residues 173–371), which is predominantly α -helical. The putative activation loop of each monomer extends into the dimer related subunit to form the interface (residues 243–255). The protein is represented by cartoon and fisetin is shown in green. Fisetin binds at the ATP binding pocket located on the hydrophobic cleft between the N- and C-terminal domains. The enlarged view of the active site illustrates disposition and hydrogen bonding interactions (in yellow) between the catechol moiety of fisetin and residues Gly100 and Gly98 involved in molecular recognition. (B) Binding modes of fisetin within the putative binding pockets of mTOR protein (*right, middle, left*). Fisetin binds to three sites on residues of helices $\alpha 2$ and $\alpha 3$.

Fig.7. Fisetin binds with higher affinity to p70S6K than mTOR but has little affinity for AKT (A,B&C) Competition binding assay for AKT, mTOR and p70S6K: The ability of fisetin to compete with DNA tagged-AKT, -mTOR and -p70S6K for active-site directed immobilized, ligand was measured via qPCR as a function of unlinked fisetin concentration. An 11-point 3-fold serial dilution of fisetin was prepared in 100% DMSO at 100x final test concentration and diluted to 1x in the assay. Kds were determined using a top concentration of 100,000nM. A value of 40,000nM indicates that the compound affinity for the kinase cannot be measured at the range of compound concentrations tested (0–100,000nM). (A) Matrix of Kds for AKT, mTOR and p70S6K, where CDC2L1 served as the negative control. Raw data of competition kinase binding assay for AKT, mTOR and p70S6K provided in Table 3 (B) Schematics showing the principle of the experiment (C) Representative curves and binding constants of duplicate experiments for each of the four kinases AKT, mTOR, p70S6K and CDC2L1 (negative control). The amount of kinase measured by qPCR (signal; y-axis) is plotted against the corresponding compound concentration in nM in log₁₀ scale (x-axis). Data points marked with an “x” were not used for Kd determination.

[A]

Kinase	Fisetin IC ₅₀ (M)	Rapamycin IC ₅₀ (M)	Staurosporine IC ₅₀ (M)
mTOR	6.71E-06	x	ND
mTOR + FKBP12	6.05E-06	3.58E-08	ND
p70S6K	5.10E-06	ND	5.27E-10
AKT1	1.44E-05	ND	8.60E-09

[B] Compound IC₅₀ Data for mTOR/FRAP1

Conc (M)	% mTOR Activity Fisetin	% mTOR Activity Rapamycin
1.00E-04	-0.67	122.42
3.33E-05	-0.78	101.34
1.11E-05	39.25	95.66
3.70E-06	74.03	91.16
1.23E-06	91.45	98.01
4.12E-07	105.12	103.20
1.37E-07	101.31	103.57
4.57E-08	109.81	109.98
1.52E-08	105.47	111.88
5.08E-09	113.63	112.92
DMSO	97.59	102.84

[C]

Fisetin (M)	% Activity
1.00E-04	-2.48
3.33E-05	-3.45
1.11E-05	37.42
3.70E-06	72.81
1.23E-06	95.58
4.12E-07	103.77
1.37E-07	108.53
4.57E-08	115.75
1.52E-08	116.50
5.08E-09	105.16
DMSO	102.72

Rapamycin (M)	% Activity
1.00E-04	62.59
3.33E-05	62.24
1.11E-05	44.88
3.70E-06	43.81
1.23E-06	45.98
4.12E-07	47.63
1.37E-07	51.36
4.57E-08	71.61
1.52E-08	88.00
5.08E-09	94.64
DMSO	100.55

[D]

Fisetin (M)	% p70S6K Activity
1.00E-04	1.29
3.33E-05	3.07
1.11E-05	11.31
3.70E-06	74.89
1.23E-06	109.86
4.12E-07	105.76
1.37E-07	106.18
4.57E-08	103.51
1.52E-08	109.45
5.08E-09	108.83
DMSO	99.56

Staurosporine (M)	% p70S6K Activity
2.00E-05	0.78
5.00E-06	0.31
1.25E-06	1.96
3.13E-07	1.54
7.81E-08	1.93
1.95E-08	2.71
4.88E-09	5.88
1.22E-09	28.06
3.05E-10	65.63
7.63E-11	98.06
DMSO	100.44

[E]

Fig.8. Fisetin mediated inhibition of p70S6K and mTOR kinase activities occurs at a lower dose than AKT

(A,B,C&D) *Kinase activity assay for AKT, mTOR and p70S6K* Fisetin was tested against mTOR, mTOR-FKBP12 complex, p70S6K and AKT alongwith reference compounds rapamycin and staurosporine. Fisetin and rapamycin were tested in 10-dose IC₅₀ mode with 3-fold serial dilution starting at 100 μ M. Staurosporine was tested in 10-dose IC₅₀ mode with 4-fold serial dilution starting at 20 μ M. Reactions were carried out at 30 μ M ATP. (A) % activity relative to DMSO controls are tabulated, where 'x' indicates no inhibition, or compound activity that could not be fitted to an IC₅₀ curve. 'ND' indicates compound not tested against the kinase (B) Representative curve of % kinase activity for mTOR with fisetin and rapamycin (*left*), raw data with serial dilution and % activity (*right*) (C) Representative curve of % kinase activity for mTOR-FKBP12 complex with fisetin and rapamycin (*bottom panel*), raw data with serial dilution and % activity (*top panel*) (D) Representative curve of % kinase activity for p70S6K with fisetin and staurosporine (*bottom panel*), raw data with serial dilution and % activity (*top panel*). (E) Representative curve of % kinase activity for AKT with fisetin and staurosporine (*bottom panel*), raw data with serial dilution and % activity (*top panel*).

Table 1

Fisetin (μM)	Assay points (days)	Total tissues (n=2/assay point)
0	4, 8, 12, 16	8
40	4, 8, 12, 16	8
80	4, 8, 12, 16	8

Table 2

No.	Sex	Age	Organ	Pathology diagnosis	Stage
1.	M	35	Skin	Normal skin tissue	-
2.	F	77	Skin	Epithelioid melanoma left sole	II
3.	M	36	Skin	Balloon cell melanoma chest wall	II
4.	M	60	Skin	Sarcomatoid melanoma buttock	III
5.	F	42	Skin	Balloon cell melanoma thigh	II
6.	M	60	Skin	Sarcomatoid melanoma buttock	III

Table 3

Fisetin (nM)	AKT	mTOR	p70S6K1
0	4.82E-09	3.31E-09	1.90E-07
1.69	5.08E-09	3.43E-09	9.75E-08
5.08	4.41E-09	4.49E-09	2.14E-07
15.2	5.97E-09	3.40E-09	1.82E-07
45.7	4.77E-09	5.34E-09	1.93E-07
137	5.11E-09	3.80E-09	1.73E-07
412	4.82E-09	3.40E-09	1.20E-07
1230	6.47E-09	3.74E-09	6.14E-08
3700	5.62E-09	3.34E-09	2.21E-08
11100	6.52E-09	1.63E-09	6.01E-09
33300	3.79E-09	8.90E-10	1.59E-09
100000	2.73E-09	7.30E-10	1.03E-08
0	3.60E-09	4.27E-09	2.04E-07
1.69	4.04E-09	5.21E-09	2.33E-07
5.08	3.82E-09	4.50E-09	2.30E-07
15.2	3.83E-09	5.10E-09	2.09E-07
45.7	3.98E-09	4.00E-09	1.71E-07
137	3.08E-09	4.34E-09	2.72E-07
412	4.38E-09	4.27E-09	1.37E-07
1230	4.34E-09	3.45E-09	1.53E-07
3700	4.42E-09	2.96E-09	2.85E-08
11100	4.66E-09	1.91E-09	7.42E-09
33300	3.41E-09	1.12E-09	1.95E-09
100000	2.18E-09	5.80E-10	6.47E-09