HHS Public Access

Author manuscript

Cancer Lett. Author manuscript; available in PMC 2016 October 28.

Published in final edited form as:

Cancer Lett. 2015 October 28; 367(2): 173–183. doi:10.1016/j.canlet.2015.07.030.

Dietary flavonoid fisetin binds to β-tubulin and disrupts microtubule dynamics in prostate cancer cells

Eiman Mukhtar[a], **Vaqar Mustafa Adhami**[a], **Mario Sechi**[b], and **Hasan Mukhtar**[a,*]

[a]Department of Dermatology, University of Wisconsin-Madison, 1300 University Avenue, 4385 Medical Sciences Center, Madison, WI 53706, USA

[b]Department of Chemistry and Pharmacy, University of Sassari, Via Vienna 2, 07100 Sassari, Italy

Abstract

Microtubule targeting based therapies have revolutionized cancer treatment; however, resistance and side effects remain a major limitation. Therefore, novel strategies that can overcome these limitations are urgently needed. We made a novel discovery that fisetin, a hydroxyflavone, is a microtubule stabilizing agent. Fisetin binds to tubulin and stabilizes microtubules with binding characteristics far superior than paclitaxel. Surface plasmon resonance and computational docking studies suggested that fisetin binds to β-tubulin with superior affinity compared to paclitaxel. Fisetin treatment of human prostate cancer cells resulted in robust up-regulation of microtubule associated proteins (MAP)-2 and -4. In addition, fisetin treated cells were enriched in α-tubulin acetylation, an indication of stabilization of microtubules. Fisetin significantly inhibited PCa cell proliferation, migration, and invasion. Nudc, a protein associated with microtubule motor dynein/dynactin complex that regulates microtubule dynamics, was inhibited with fisetin treatment. Further, fisetin treatment of a P-glycoprotein overexpressing multidrug-resistant cancer cell line NCI/ADR-RES inhibited the viability and colony formation. Our results offer *in vitro* proof-of-concept for fisetin as a microtubule targeting agent. We suggest that fisetin could be developed as an adjuvant for treatment of prostate and other cancer types.

Keywords

Fisetin; Microtubules; Prostate cancer; Proliferation; Migration

Introduction

Microtubules play an essential role in the intracellular cytoskeletal framework. They are polymers made of repeating αβ-tubulin heterodimers that are involved in cell division, migration, signaling, and intracellular trafficking which also makes them important in cancer cell proliferation and metastasis [1]. The dynamics of microtubule polymerization are central to their biological function. Polymerization dynamics allows microtubules to adapt to

*Corresponding author. Tel.: +1 608 263 3927; fax: +1 608 263 5223. hmukhtar@wisc.edu (H. Mukhtar).

Conflict of interest

The authors declare no conflicts of interest, financial or otherwise.

spatial arrangements that can change rapidly in response to cellular needs and to perform mechanical work. Correspondingly, microtubule dynamics as well as associated signaling pathways are important targets for novel anti-cancer and anti-neurodegenerative therapies. Thus, microtubule targeting agents (MTAs) have become a very successful class of cancer drugs that have proven their potency and efficacy against many cancer types including prostate cancer (PCa). Despite their success, resistance and severe side effects restrict their clinical use [2].

One of the major problems in chemotherapy is multidrug resistance (MDR) against anti-cancer drugs. ATP-binding cassette (ABC) transporters are a family of proteins that mediate MDR via ATP-dependent drug efflux pumps. Although many MDR inhibitors have been identified, none of them have proven clinically useful without side effects. Therefore, novel approaches are required to identify nontoxic MDR inhibitors that can inhibit or circumvent MDR. Many MTA are substrates of P-glycoprotein (P-gp), a 170 kDa protein encoded by human *ABCB1* gene. This gene belongs to a broad spectrum ATP-dependent efflux pump that reduces drug efficacy and higher doses of these drugs are required to achieve adequate intracellular concentration in cancer cells [3–5]. Therefore, inhibition of P-gp function represents a logical approach to overcome MDR in cancer chemotherapy.

There has been substantial research effort to investigate the use of natural products, with good safety profile, to inhibit cancer and many other diseases [6,7]. Fisetin (3,3′,4′,7-tetrahydroxy flavone) is a flavonol present in the diet at concentrations ranging from 2 to 160 μg/g, and at much higher concentrations as an ingredient in nutritional supplements. Fisetin has anti-proliferative, apoptotic and antioxidant activities, and is under study for its chemopreventive/chemotherapeutic effects against several cancers, as well as for its neuroprotective properties [8–10]. We report here that fisetin binds to β-tubulin and disrupts microtubule dynamics and is able to overcome drug resistance in the NCI/ADR-RES cell line.

Materials and methods

Fisetin and 4′,6-diamidino-2-phenyindole (DAPI), and paclitaxel were purchased from Sigma (St. Louis, MO); PC-3, DU-145 cells and dimethyl sulfoxide (DMSO) from ATCC (Manassas, VA). NCI/ADR-RES cell line was obtained from the DTP Human Tumor Cell Line Screen (Developmental Therapeutics Program, NCI, Frederick, MD). RPMI-1640 media, fetal bovine serum, penicillin, and streptomycin from Mediatech, Inc (Manassas, VA); α-tubulin and acetylated α-tubulin from Santa Cruz (Dallas, TX); FITC-conjugated goat anti-mouse antibody and anti-mouse, anti-rabbit secondary antibody conjugated to horseradish peroxidase and BrdU Cell Proliferation Assay Kit from Cell Signaling (Danvers, MA); β-tubulin, MAP-2, MAP-4, NudC and GAPDH from Abcam (Cambridge, MA); antifade agent Prolong Gold-DAPI from Life Technologies, Inc. (Grand Island, NY); pure tubulin, OD based-Porcine (BK006P) proteins from Cytoskeleton (Denver, CO); Chemicon cell invasion assay kit from Millipore (Billerica, MA); FITC-dUTP from BD Pharmingen™ (San Jose, CA); anti-GST antibodies from GE Healthcare Life Sciences (Piscataway, NJ). All chemicals were stored as aliquots of 100 mM stock solutions in DMSO at −20 °C.

Cell culture

Human PCa cell lines (PC-3, DU-145) and NCI/ADR-RES cell were cultured in RPMI 1640 medium supplemented with 10% FBS (v/v) and penicillin (100 U/ml)/streptomycin (100 μg/ml). Cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2.

In vitro microtubule polymerization assay

Tubulin proteins were suspended in G-PEM buffer plus 3% glycerol in the absence (control) or presence of fisetin or paclitaxel at 10 μM concentration at 4 °C. Polymerization was followed by measuring the increase in fluorescence over a 60 minute period at 37 °C.

Surface plasmon resonance (SPR) binding assays

Binding experiments were performed using a Biacore T-200 instrument (Biacore, Uppsala, Sweden) at 25 °C. Human β-tubulin full length protein (1 aa–444 aa, 76 kDa including GST tag), 6000 RU (response units) of the protein, was directly immobilized on flow cell 2 by amine coupling method using N-ethyl-N-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) in water. The same number of RU of GST alone was immobilized on flow cell 1 for reference subtraction. GST-antigen was flowed over the chip (CM5, GE certified) at variable concentrations in 10 mM sodium acetate buffer (pH 4.0), with a low rate of 1 μl/min. Binding of antigen to the anti-GST antibodies was monitored in real time to obtain on (ka) and off (kd) rates. The equilibrium constant (KD) was calculated by steady state kinetics due to fast off rate. Both paclitaxel and fisetin stocks were prepared in 100% DMSO, and further dilutions were made in assay buffer containing 10 mM HEPES buffer (pH 7.4), 150 mM NaCl, 3 mM EDTA, 0.005% P20 (polyoxyethylenesorbitan), 1 mM CaCl2 and 5% DMSO. Scouting was performed at 4000 nM of each of the analyte. Full kinetic analysis was performed using analyte concentrations from 2000 nM to 0 (run serial dilutions, 1000, 500, 250, 125 and 0), and a flow rate of 50 μl/min.

Computational docking method

Ligand docking studies were performed using Molecular Operating Environment (MOE 2009.10). The three-dimensional structure of αβ-tubulin (at 3.5 Å resolution) used for docking experiments was downloaded from the PDB Data Bank [http://www.rcsb.org/ – PDB code: 1jff (PMID: 11700061)]. Rigid receptor-flexible ligand docking calculation was performed using the docking simulation feature MOE-dock by setting grid sizes that included the entire macromolecule. The London $\Delta G_{binding}$ scoring function that estimates the free energy of binding (in kilocalories per mole, kcal/mol) was used to rank hit docking pose candidates. Only the best scored poses generated in the docking experiments were retained and examined with MOE.

Cold-induced microtubule depolymerization

PC-3 cells were seeded on two chamber glass slides and cultured overnight. Cells were treated with fisetin (80 μM) for 24 hours, and control cells were treated with 1% DMSO. After 2 hours of treatment, glass slides were put on ice for 0–90 minutes. Cells were fixed with 4% paraformaldehyde and 0.5% glutaraldehyde for 10 min at room temperature, and then permeabilized and saturated with a solution of 0.1% Triton X100 in phosphate buffered

saline (PBS)–1% bovine serum albumin for 1 hour at room temperature. Cells were incubated overnight at 4 °C with a primary monoclonal antibody anti-α-tubulin and then incubated for 1 hour at room temperature with a mouse secondary FITC labeled antibody. Fixed cells were mounted on glass slides with antifade agent Prolong Gold-DAPI and photographed using a Nikon fluorescence microscope.

Western blot analysis

PCa cells were treated with fisetin or control for 24 hours, harvested and then lysed in RIPA buffer. A total of 30 μg of protein was mixed with Laemmli sampling loading buffer, separated by 6–15% gradient SDS-PAGE. Then the samples were transferred to nitrocellulose and incubated with primary and appropriate HRP-conjugated secondary antibodies. Proteins were detected by enhanced chemiluminescence using Bio-Rad chemi-Doc MP system.

Cell migration

Migration was studied by wound-healing assay. PC-3 cells were plated in 60 mm^2 Petri dish plates, when the cells ~90% confluent, a wound was induced by scraping a gap using a micropipette tip. The speed of wound closure was compared between fisetin 10–80 μM treated and untreated control groups. Photographs were taken 0–72 hours after wound incision.

Cell invasion assay

PC-3 cells were analyzed for invasion using the Chemicon cell invasion assay kit according to the manufacturer's protocol. Briefly, suspension of 0.5–1.0 × 10^6 cells/ml in serum-free medium was loaded into ECMatrex layer inserts and allowed to migrate to bottom of membrane for 72 hours at 37 °C. Cells were removed from the top of the inserts and those that invaded through the polycarbonate/basement membrane were fixed, stained and quantified by dissolving stained cells in 10% acetic acid (100–200 μl/well) and transfer a consistent amount of the dye/solute mixture to a 96 well plate for colorimetric reading of OD 560 nm.

BrdU cell proliferation chemiluminescent assay

PC-3 cells were analyzed for cell proliferation using BrdU cell proliferation assay kit according to the manufacturer's protocol. Briefly, cells are cultured with labeling medium that contains BrdU; this pyrimidine analog is incorporated in place of thymidine into the newly synthesized DNA of proliferating cells. After removing the labeling medium, cells were fixed and the DNA was denatured with a fixing/denaturing solution. A BrdU mouse mAb was then added to detect the incorporated BrdU. Anti-mouse IgG, HRP-linked antibody was used to recognize the bound detection antibody. Chemiluminescent reagent was added for signal development followed by measurement of OD at 425 nm.

Cell cycle analysis by flow cytometry

PC-3 cells were treated with or without fisetin (0–80 μM) for 24 hours. Then, cells were harvested and fixed and processed according to the manufacturer's protocol. Stained cells were immediately analyzed by FACS Calibur for cell cycle analysis.

Viability assay

Cells were seeded (6×10^4 cells/2 mL) in 6-well plates (24 hours) and treated with or without fisetin (0–80 μM) for 24, 48, and 72 hours. Cell viability was determined by Trypan blue assay (Bio-Rad, CA) according to the manufacturer's protocol.

Clonogenic assay

Cells were seeded (500–1000 cells/5 mL) in 60 mm^2 Petri dish plates for 24 hours. Cells were treated with or without fisetin (0–80 μM) and allowed to form colonies in 1–3 weeks. Colonies were fixed with glutaraldehyde (6.0% v/v), stained with crystal violet (0.5% w/v) and photographs were taken.

Statistical analysis of the data

Microsoft Excel software was used to calculate the mean and the standard error of the mean. The results were analyzed using a two-tailed Student's t-test to assess statistical significance and p values <0.05 were considered significant.

Results

Fisetin stabilizes microtubule assembly *in vitro*

Using an *in vitro* tubulin polymerization assay, we discovered that fisetin enhanced microtubule polymerization to a greater extent than paclitaxel under conditions that required an enhancer for microtubules to polymerize efficiently (Fig. 1A). Paclitaxel stabilizes microtubule polymer and prevents it from disassembling [11]. Our observation suggests that fisetin at similar dose has the same effect but is faster and much efficient (fisetin Vmax = 65 m OD/min vs. paclitaxel Vmax = 12 m OD/min) than paclitaxel.

To further investigate the stabilization of the microtubule network by fisetin, we evaluated microtubule resistance to cold-induced depolymerization. Control and fisetin-treated cells (80 μM) were subjected to cold for 0–90 minutes and processed for indirect α-tubulin immunofluorescence. Microtubule depolymerization was clearly evident at 30 min in control treated cells and complete depolymerization was observed at 90 min (Fig. 1B). In fisetin treated cells, microtubule depolymerization was not noticeable at 30 min, indicating an increase of microtubule network stability. At 60 and 90 minutes the microtubule network was completely disorganized and tubulin appeared to form aggregates. It was also observed that the fisetin-induced cell extensions and filopodias remained intact at least up to 90 minutes.

Fisetin interferes with microtubule assembly and physically interacts within the β-tubulin binding site

To support the assumption that fisetin direcly interacts with microtubules, we first investigated the binding between fisetin and β-tubulin by real-time interaction analysis using surface plasmon resonance (SPR) assays, which allows kinetic and affinity evaluation and determination of binding specificity between proteins and small molecules. Binding evaluations suggested that both fisetin and paclitaxel behave as specific and stable binders, thus revealing high-affinity with β-tubulin. In particular, fisetin binds to β-tubulin with a K_D value of 1.59 μM, with close affinity with respect to paclitaxels' K_D value of 2.26 μM (Fig. 2A–D).

To further characterize the putative interaction of fisetin with β-tubulin at molecular level, we computationally examined the β-tubulin pocket within the paclitaxel binding site. The docking of paclitaxel suggests a nice fitting between the dispositions of the structure in the X-ray structure (Fig. 3A–C). Top-ranked energy scoring poses of fisetin show various binding modes and tight affinity within the paclitaxel amino acid pocket (Fig. 3D–I). Calculated free binding energy (ΔG_b) for these conformations was around −16 kcal/mol (Fig. 4A and B), thus indicating good binding energies, better than that of paclitaxel which was around −6 kcal/mol.

We also examined the hypothetical binding modes and tight affinity within the amino acid pocket that includes the following residues: Val 23, Glu 27, Ala 233, Ser 236, Phe 272, Pro 274, Leu 275, Thr 276, Pro 360, Arg 369, Gly 370, Leu 371 (Fig. 4C and D). The most favorable conformation placed the α-keto-hydroxyl functional groups of the chromen-4-one ring in close proximity to the Gly 370, involving strong H-bond interaction. Other amino acid residues involved in van der Waals and hydrophobic interactions with fisetin were located near the catalytic site. Thus, further computational studies and correlation with experimental data to validate these findings are necessary.

Fisetin treatment interferes with microtubule associated proteins and cell cycle arrest

Because acetylation of α-tubulin is considered a marker of stable microtubule structure, we tested whether fisetin increased acetylated α-tubulin that correlates with increased microtubule stability. We found that treatment of PC-3 and DU145 cells with fisetin (20–80 μM) for 24 hour induces increased expression of acetylated α-tubulin in a dose dependent manner when compared with α-tubulin and β-actin as control (Fig. 5A). These results suggest that fisetin treatment stabilizes microtubules and affects the state of post-translational modification of tubulin.

To gain further insights into the actions of fisetin, we determined its effect on MAP-2 and MAP-4 expression; members of the family best known for their microtubule-stabilizing activity. We found a dose dependent increase in the expression of MAP-2 and MAP-4 in PC-3 and DU145 cells treated with fisetin (Fig. 5B). We found that increased MAP4 expression, which occurs with PC-3 (p53 null) and DU145 (mutant p53) cells, is associated with increased sensitivity to fisetin.

Nuclear migration protein NudC, a protein that associates with the microtubules motor dynein/dynactin complex and regulates microtubule dynamics, plays an essential role in mitosis and cytokinesis. We tested whether fisetin modulates the expression of the NudC protein in PCa cells. We found that fisetin downregulated the expression of NudC protein in PC-3 and DU145 cells (Fig. 5C), which is correlated with the inhibition of the cell growth and proliferation.

Induction of mitotic arrest is a general effect of tubulin-targeting agents. Therefore, we tested whether fisetin induces cell cycle arrest at the G2/M phase. Flow cytometry analysis suggested that cells in G2/M increased from 31% in control to 46% at 80 μM of fisetin treatment at 24 hours (Fig. 5D). The data presented suggest that fisetin blocks mitosis and inhibits cell proliferation by perturbing microtubule assembly dynamics.

Fisetin treatment inhibits PCa cell proliferation, invasion and migration, processes that are closely regulated by the cytoskeleton

Because defects in cell-cycle regulation are a fundamental feature of cancer pathogenesis, we investigated whether fisetin inhibits the proliferation of the PCa cells. Fisetin inhibited cell proliferation significantly ($p = 0.04103$) at 20 μM concentration (Fig. 6A). We also found decreased expression of proliferating cell nuclear antigen (PCNA) (Fig. 6B) and Ki67 (Fig. S1B), markers of cell proliferation in PC-3 cells treated with. Thus, these results suggest fisetin as a potent proliferation inhibitor and a promising chemotherapy agent. We investigated if PC-3 cells could resume proliferation following removal of fisetin treatment. If fisetin-treated cells retained proliferation capacity, total cell count should increase when further cultured in drug free media. As shown in Fig. S1B, we found that fisetin impaired PC-3 cell proliferative potential for up to 5 days in drug free media.

Next, we investigated the effect of fisetin on the invasion of PCa cells after 72 hours of incubation. As shown in Fig. 6C, fisetin inhibited the cells ability to invade through a membrane in the presence of 20 μM concentration ($p = 0.05$). From these observations we conclude that fisetin inhibits cell migration significantly at 20 μM *in lieu* of its effects on mitosis at the same concentration.

Over the past decade, microtubules have revealed their pivotal role in cell migration and are required for both directional and random movements. It is perhaps not surprising, therefore, to predict the effect of fisetin as microtubule modulating agent on cell migration. We found that fisetin (10–80 μM) inhibited PCa cells migration considerably in a time dependent manner (Fig. 6D). Dose–response measurement further showed that low drug concentrations inhibited cell migration. These findings led us to conclude that fisetin is indeed a microtubule interfering agent that suppresses microtubules which in turn led to reduction in cell migration. Further studies are warranted to elucidate the possibility that cell motility is not affected by some other actions of the fisetin. Fisetin treatment enhanced and delocalized microtubule polymerization throughout the cell, decreased proliferation, and increased apoptosis. Subset of cells became binucleated and multinucleated, suggesting a failure of cytokinesis (Fig. S1C).

Fisetin treatment inhibits the viability and colony formation in a P-gp-overexpressing NCI/ADR-RES cell line

We examined the susceptibility of a P-gp-overexpressing NCI/ADR-RES cell line to treatment with fisetin. NCI/ADR-RES cells were treated with fisetin (0–80 μM) for 72 hours and cell viability was determined by Trypan blue staining. Fisetin treatment decreased cell viability in a concentration-dependent manner (Fig. 7A). We next used a clonogenic assay for studying the effectiveness of fisetin on the survival and proliferation of NCI/ADR-RES cells. As shown in Fig. 7B, fisetin treatment significantly inhibited the colony formation of NCI/ADR-RES cells at 40–80 μM concentration. In addition, by using western blot analysis we found that fisetin downregulated P-gp protein in NCI/ADR-RES cells (Fig. 7C).

Discussion

Natural agents that target microtubules and disrupt the normal function of the mitotic spindle have an excellent selectivity against cancer cells and have been proven to be the key components of chemotherapeutic regimens for various solid tumors. Thus, development and discovery of new drugs, and exploring new treatment strategies that reduce side effects and circumvent drug resistance could provide more effective therapeutic options for patients with cancer. A better approach is to identify small molecules that have multiple biological activities with less toxicity and that could also counteract resistance.

To investigate the putative interaction between fisetin and β-tubulin, we assumed to locate fisetin within the taxol binding pocket on the basis of (1) a similar mechanism of action and (2) assuming that “most of the stabilizing agents bind to the same or to an overlapping taxoid-binding site on β-tubulin, which is located on the inside surface of the microtubule” [12,13]. In the literature there is a consolidated opinion about a common binding site between paclitaxel and the epothilones (these compounds competitively inhibit [3H]-paclitaxel binding to microtubules), thus we performed an interesting study on the binding site of paclitaxel and an Epothilone analog desoxyepothilone B (dEpoB) [14]. We performed docking experiments using the three-dimensional structure of β-tubulin (at 3.5 Å resolution), downloaded from the PDB Data Bank (http://www.rcsb.org/ – PDB code: 1jff) [15]. We examined the hypothetical binding modes within the amino acid pocket that includes the following residues: Val 23, Glu 27, Ala 233, Ser 236, Phe 272, Pro 274, Leu 275, Thr 276, Pro 360, Arg 369, Gly 370, Leu 371 (Fig. 4C and D). Fisetin did not interact with A231T and Q292E – mutations found for Epothilone analog desoxyepothilone B (dEpoB). Therefore, docking analysis indicated a common binding site between paclitaxel and fisetin. However, the precise binding sites of fisetin with β-tubulin remain to be established.

Although microtubules generally function as highly dynamic polymers, α-tubulin acetylation is associated with unusually stable microtubules and found in the dense networks and bundles of microtubules [16]. Fisetin increases the expression of the acetylated α-tubulin. This finding is in agreement with prior studies which suggest that acetylated α-tubulin, under depolymerizing condition, is more stable than the majority of cytoplasmic microtubules. Also, the α-tubulin of 3T3 and HeLa cells becomes acetylated in the presence of taxol [17].

MAP-2 and MAP-4 members of microtubule associated proteins family that are best known for their microtubule-stabilizing activity play important roles in regulating microtubule network [18]. MAPs also play a critical role in stabilizing microtubules that face the front edge. Stable microtubules accumulate posttranslational modifications; these posttranslational modifications, such as acetylation, detyrosination, glutamylation, and glycilation, are used as markers of stabilized microtubules, which are relatively long-lived microtubules that also appear more resistant to cold or to microtubule-depolymerizing drugs as reported by others [19]. MAP-4 stabilizes polymerized microtubules by binding to the negatively charged C-terminus of α- and β-tubulin [20]. We found increased expression of MAP-2 and MAP-4 with fisetin treatment in a dose dependent manner, consistent with stabilization of microtubule by fisetin. MAP-4, which is expressed in many cell types, regulates cell cycle progression. Previous studies indicate that the cyclin B/MAP-4 interaction plays a crucial role in targeting mitotic kinases to cytoskeletal substrates and in regulating spindle assembly and dynamics during cell division [21]. Because mutations in p53 change the sensitivity of tumor cells to chemotherapeutic drugs, the study found that increased MAP4 expression, which occurs with transcriptionally silent p53, is associated with increased sensitivity to paclitaxel and decreased sensitivity to vinca alkaloids [22]. Therefore, p53-regulated changes in expression of MAP-4 would alter microtubule dynamic stability and could thus influence the sensitivity to drugs whose mechanism of action affected the polymerization or depolymerization of these critical cellular components. Since MAP-4 stabilizes polymerized microtubules, overexpression of this gene provides a possible mechanism to explain the altered sensitivity to MTA in the presence of mutant/null p53. In addition, our results are in agreement with the results of others who observed that microtubules in cells transfected with microtubule-associated proteins become enriched in acetylated tubulin as well as the stabilization of microtubules [23]. These findings support our earlier result that showed fisetin stabilized microtubules against cold-induced depolymerization and also increased α-tubulin acetylation.

Nuclear migration protein NudC, a protein associated with the microtubule motor dynein/dynactin complex that regulates microtubule dynamics, also known as nuclear distribution protein C homolog, plays a role in neurogenesis and neuronal migration. It is necessary for correct formation of mitotic spindles and chromosome separation during mitosis, also, for cytokinesis and cell proliferation. Elevated NudC levels correlate with proliferative status of various cell types and is essential for colony growth and viability [24]. We found that fisetin downregulated the expression of NudC protein in PC-3 and DU145 cells in a dose-dependent manner, correlating with the inhibition of the cell growth. Although increased level of NudC may be required for cell proliferation, other studies reported that overexpression of NudC results in inhibition of cell division. Thus, a proper level of NudC is critical for cell cycle progression (11).

Our data suggest that fisetin blocks mitosis and inhibits cell proliferation by perturbing microtubule assembly dynamics (Fig. 8). Our results are in agreement with others who suggested that most of the flavonoids including fisetin demonstrated antiproliferative and cell cycle arresting properties in human PCa cell lines *in vitro* [25].

Genes that involved in cell growth and proliferation have been observed to be expressed at high levels in almost all tumor microarray datasets. This is consistent with studies where normal tissue and tumor samples are compared by microarray analysis, the biggest differences most often occur in the expression levels of genes that control cell proliferation [26]. Our findings showed that fisetin inhibit the PC-3 cell proliferation and invasion at 20–80 μM concentrations, these outcomes are consistent with the action of the drugs like paclitaxel that enhance microtubule assembly [27].

Cell migration is a dominant process in the development and maintenance of multicellular organisms, for instance, wound healing and immune responses require the proper arranged movement of cells in a particular direction to specific locations. Errors during this process, however, have serious consequences including tumor formation and metastasis [28]. These findings led us to conclude that fisetin is indeed a microtubule interfering agent that suppresses the microtubules which in turn led to reduction in cell migration. A parallel conclusion was previously reported that low drug concentrations in various cell lines do not affect mitosis yet inhibit motility [29], as well as by experiments showing that inhibition of cell migration by antimitotic drugs is due to their suppression of microtubule dynamics rather than to some other drug effects [30,31].

The efficacy of cancer chemotherapy is hindered by tumor cells exhibiting MDR, caused by changes in the level or activity of membrane transporters that mediate energy-dependent drug efflux and of other proteins that affect drug metabolism and/or drug action. Several approaches are introduced to inhibit mechanisms that are involved in the regulation of MDR transporters. MDR protein gene expression in tumor cells is induced upon treatment with cytotoxic drugs, while this gene expression is inhibited by several pharmacological inhibitors that affect the signaling pathways [32]. It was demonstrated that taxol stimulated MDR1 expression via its direct interaction with and activation of the nuclear steroid receptor which led to increased drug resistance and faster drug clearance [33]. As we shown in Fig. 6C, downregulation of P-gp protein involved in cancer cell resistance using fisetin may provide an efficient approach to overcome MDR1. This findings is in a parallel with a study that use antisense oligonucleotide technologies, which suggest an alternative and more specific way to cope with MDR by downregulation of ABC transporter proteins and enzymes using antisense oligonucleotides than the use of conventional MDR inhibitors [34].

Conclusion

Drugs that affect microtubule dynamics are among the most effective anticancer agents in routine clinical use. Although the vast majority of known MT-stabilizing agents are structurally complex, the data reported here provide the first evidence that fisetin is a microtubule stabilizing agent far superior than paclitaxel. In terms of therapy, almost all cytotoxic chemotherapeutic drugs arrest dividing cells by causing DNA damage or targeting products of proliferation-signature genes, as we observed in this study. We provide the first evidence supporting that fisetin could be further developed as proliferation and migration inhibition agent. As our understanding of drug action and resistance mechanisms has increased, we can exploit these to design strategies that overcome resistance, thus improving the efficacy of MTAs for the treatment of cancer and other disease. We suggest that fisetin

could be used for the treatment of advanced PCa alone or as an adjuvant with other chemotherapy drug.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the United States Public Health Service Grants RO1 CA 160867 and RO1 CA 160867 S1 (to H. Mukhtar). M.S. gratefully acknowledges the Regione Autonoma della Sardegna for financial support grant CRP-25920 (annualità 2010) within the frame of “Legge regionale n° 7/2007”, and also thanks the Precision Antibody (Columbia, MD, USA) for their helpful technical assistance. We would like to thank the Biological Testing Branch of National Cancer Institute for providing the NCI/ADR-RES cell line.

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.canlet.2015.07.030.

Abbreviations

MTA	microtubule targeting agents
PCa	prostate cancer
MAP	microtubule associated proteins
P-gp	P-glycoprotein
MDR	multidrug resistance
ABC	ATP-binding cassette

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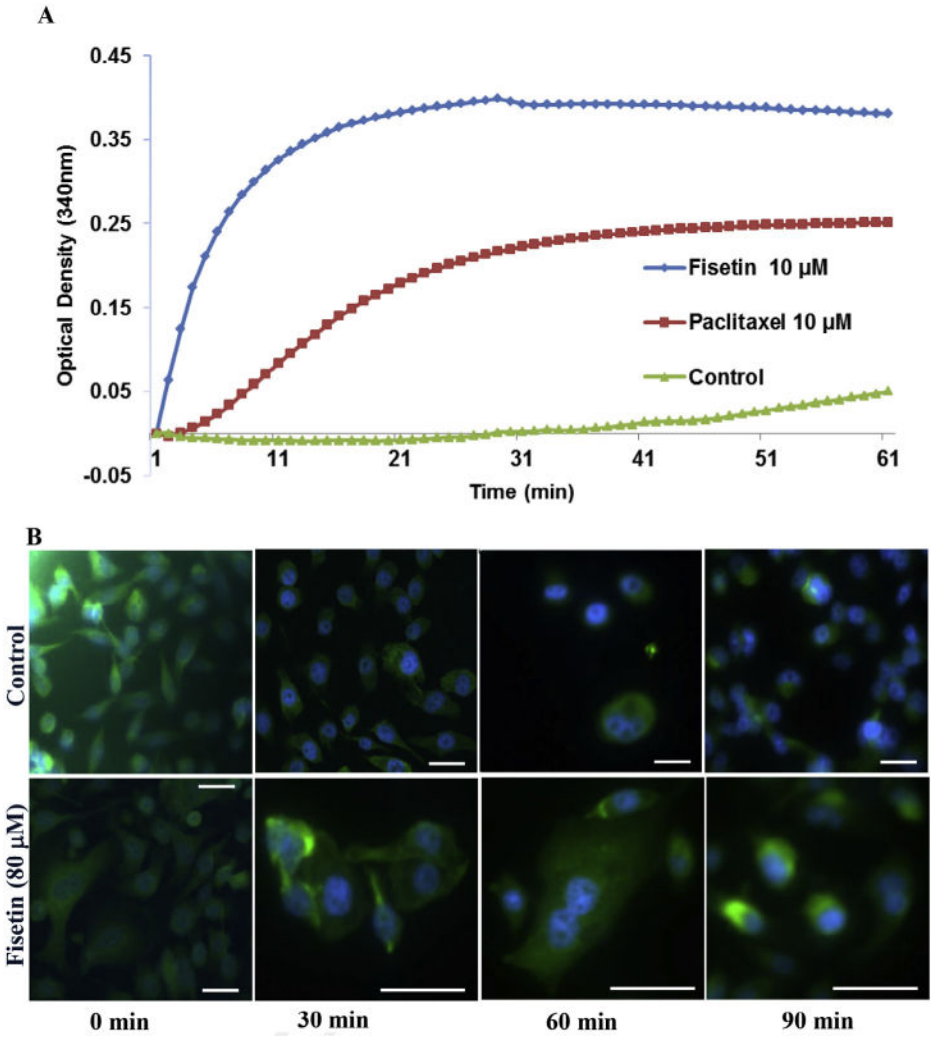

Fig. 1.

Effect of fisetin on microtubule assembly *in vitro*. (A) Graph of microtubule polymerization in the presence of fisetin (10 μM), paclitaxel (10 μM), and control. Tubulin polymerization was measured by spectrophotometer at 340 nm every 1 min for 60 min. Data form a typical experiment performed three times with similar results. (B) Representative immunofluorescence photomicrographs of PC-3 cells incubated with DMSO (Control) and fisetin (80 μM) for 0, 30, 60, 90 minutes. The microtubule network was analyzed with the Nikon confocal system. Microtubule networks (green fluorescence), nuclei labeled with DAPI (blue fluorescence). Scale bars, 25 μm and 50 μM. Images are representative of three independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

A

Protein	Analyte	KD (M)	Conc. (nM)	Chi^2 (χ^2)
Beta-tubulin	Fisetin	1.59×10^{-6}	0-2000	0.0216

B

Ligand	Analyte	KD (M)	Conc. (nM)	Chi^2 (χ^2)
Beta-tubulin	Paclitaxel	2.26×10^{-6}	0-2000	0.0118

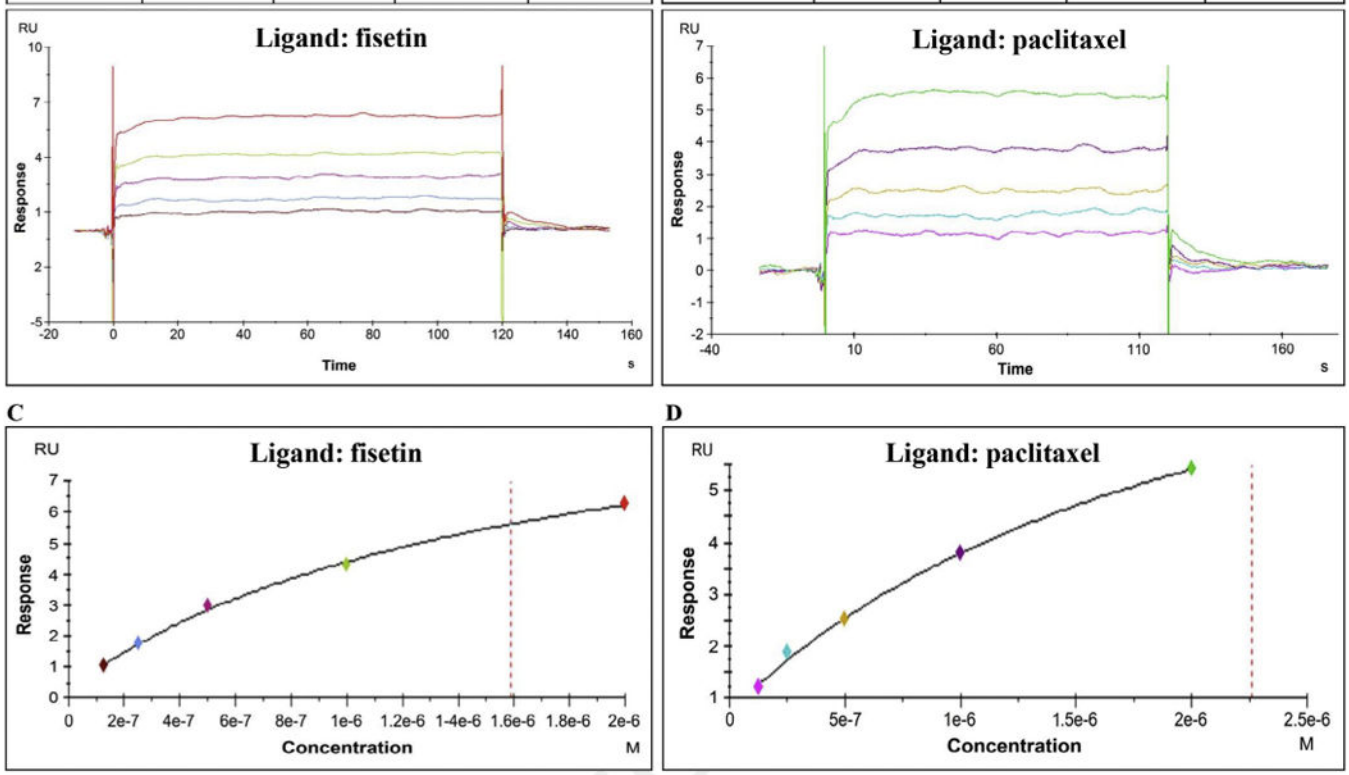

Fig. 2.

Fisetin physically binds to β-tubulin protein. (A–D) Direct binding characteristics of fisetin and paclitaxel with β-tubulin protein were analyzed using SPR measurements in a Biacore T-200 system. The β-tubulin was directly immobilized in the sensor chip by amine coupling and ligands fisetin or paclitaxel were flowed over the protein-coated chip at different concentrations (2000, 1000, 500, 250, 125 and 0 nM). Steady state kinetics were used for determining the KD due to fast off rate. Chi square (χ^2) analysis was carried out between the actual sensorgram (colored lines) and the sensorgram generated from the BI Analysis software (black line) to determine the accuracy of the analysis. χ^2 value below 1 is highly significant (highly accurate). (A, B) Representative sensorgrams of binding to β-tubulin are shown for fisetin (A) and paclitaxel (B). Data for affinity evaluation (K_Ds) were obtained from a concentration dependent binding curve for the interaction of each ligand with tubulin (A and B, for fisetin and paclitaxel, respectively). RU, resonance units. Data shown are representative of three independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

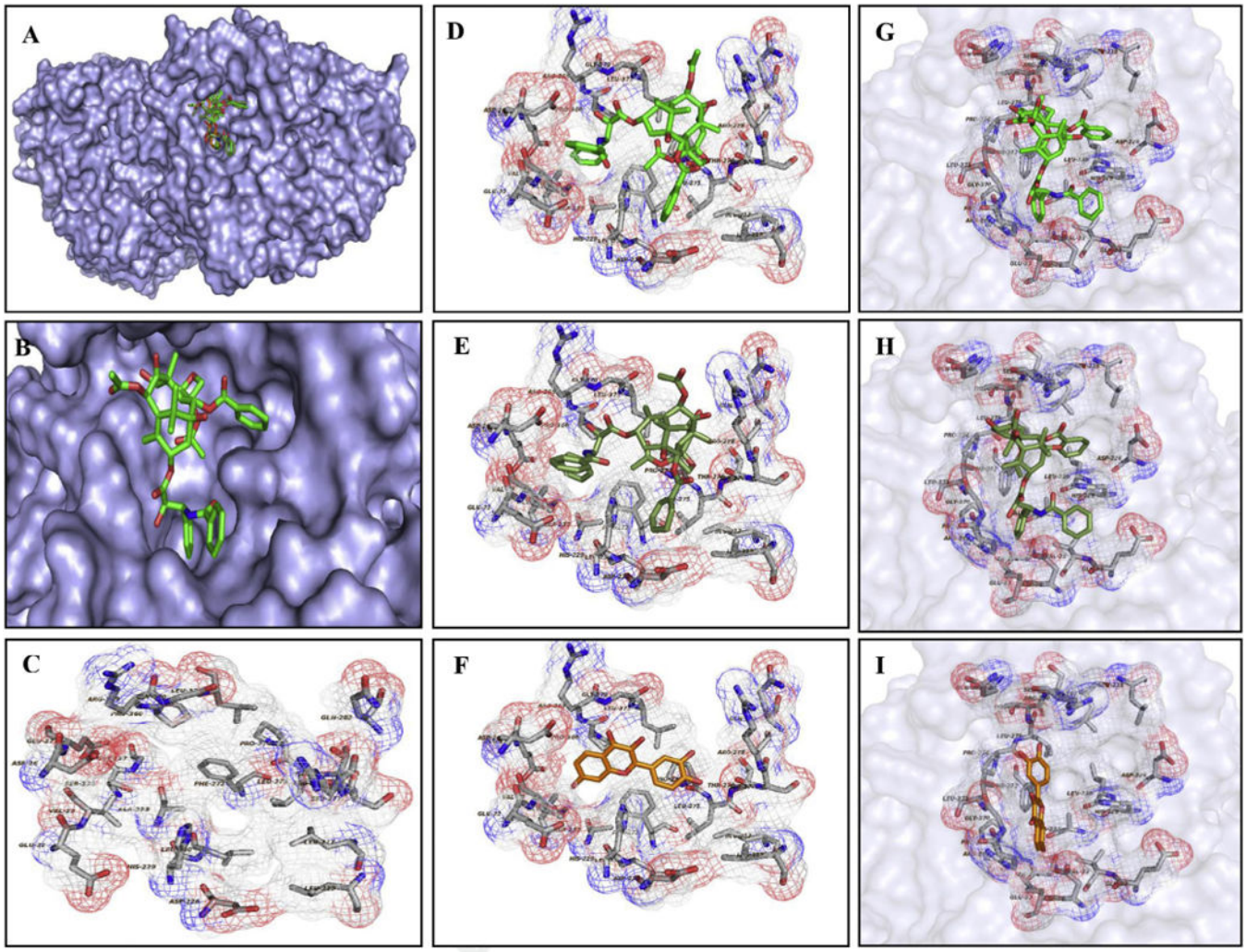

Fig. 3.

Fisetin and taxol interact within the β-tubulin binding pocket. (A) X-Ray co-crystal structure of β-tubulin and taxol. (B) Superimposition of taxol and of fisetin (both from X-ray structure and from docking calculation) onto the β-tubulin biding site. (C) Amino acid binding pocket of taxol on β-tubulin. (D–G) Representative view of taxol derived from X-ray. (E–H) Representative view of taxol derived from docking. (F–I) Representative view of fisetin on the amino acid binding pocket on β-tubulin domain.

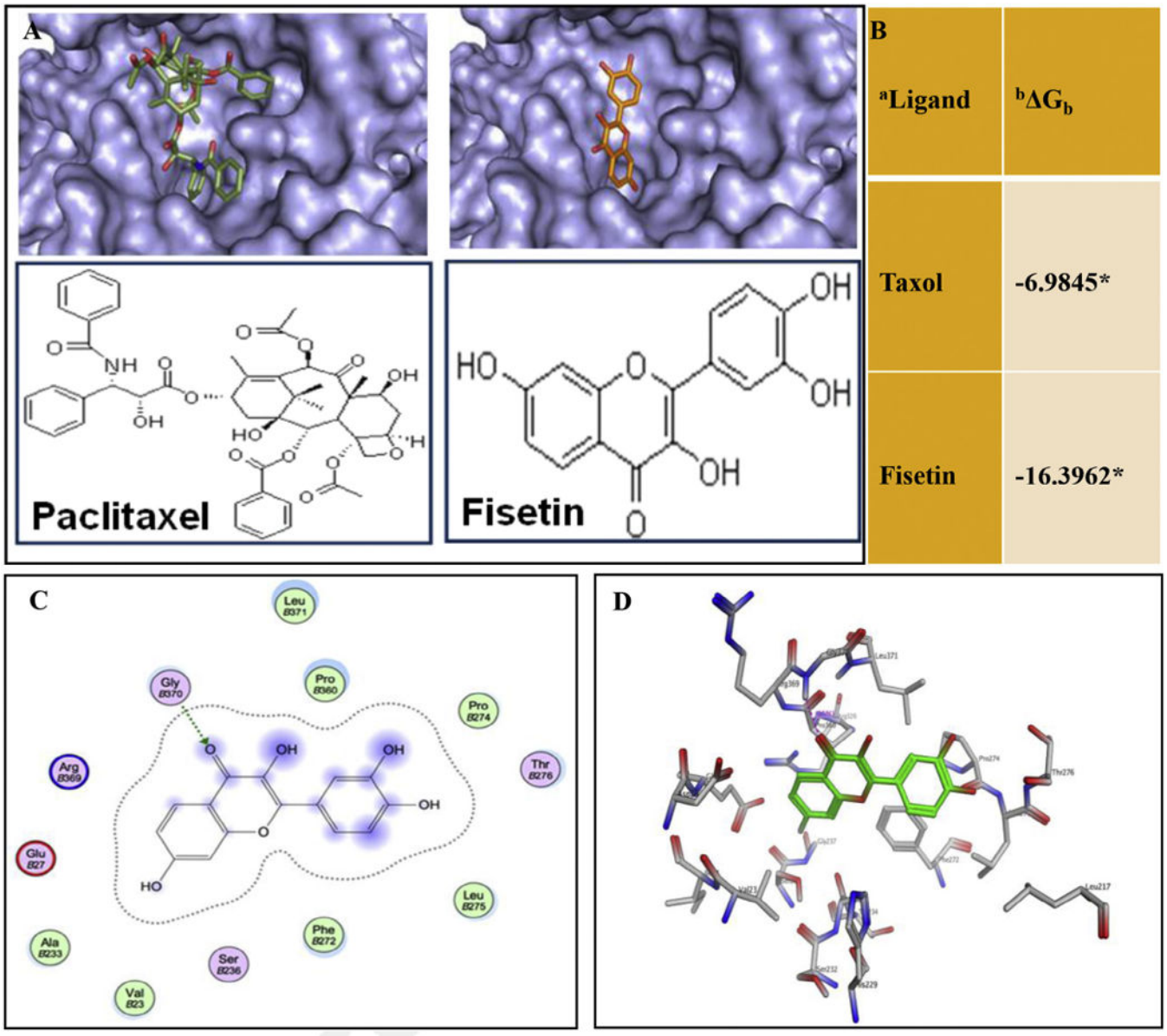

Fig. 4.

Fisetin and paclitaxel physically binds to β-tubulin protein. (A) Hypothetical model of the β-tubulin protein in complex with paclitaxel (left) and fisetin (right). *In silico* molecular modeling studies using AutoDock and 1jff.pdb as the starting receptor docking site showed that fisetin binds to β-tubulin. (B) Summary of docking results for the top-ranked poses of taxol and fisetin bound to target protein β-tubulin. [a]Ligand = top-ranked pose number; [b]ΔG_b = final score of free binding energy ($\Delta G_{binding}$) for a representative conformer (*); energy values are expressed in kcal/mol. Data form a typical experiment performed two times with similar results. (C) Two-dimensional interaction map for fisetin onto the β-tubulin protein binding site. Dashed arrows denote H-bonds bonding (green for H-bonds formed with the residue side chain), and the arrowheads indicate their direction (i.e., the donor is at the base of the arrow, whereas the acceptor is at the head). Amino acid residues (circles) are shown

as follow: (a) hydrophobic residues (green interior), (b) polar residues (light purple), (c) basic residues (blue ring), (d) acidic residues (red ring). Differences in solvent accessible surface area for fisetin ligand and for receptor residues are plotted as a blue smud and a turquoise halo, respectively. The border of the binding pocket is marked by a dashed gray line. (D) Hypothetical disposition of the best energy fisetin docking pose within the putative β-tubulin binding pocket. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

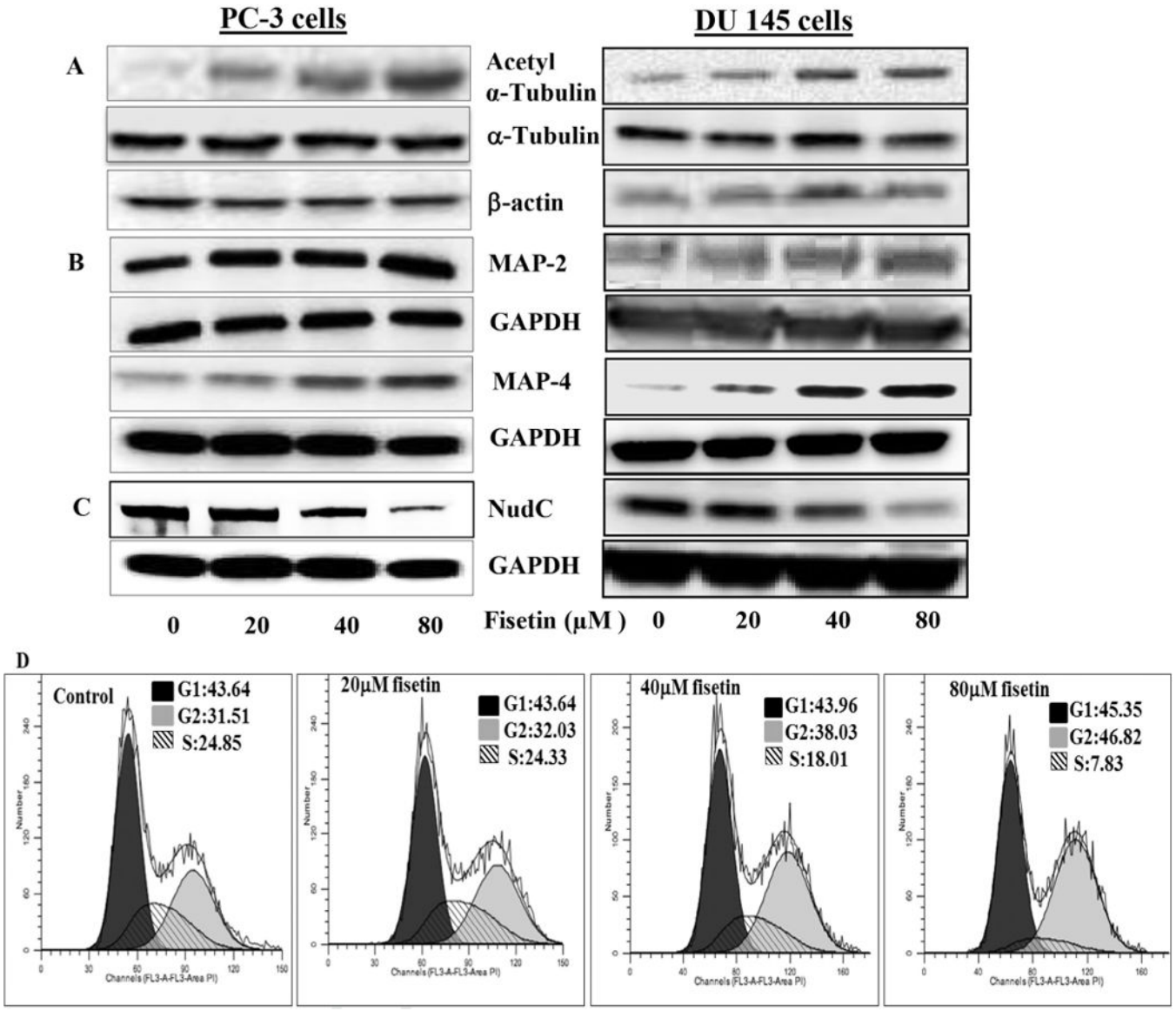

Fig. 5.

Effect of fisetin treatment on proteins associated with microtubule organization and cell cycle. (A–C) Representative blots showing the effect of fisetin treatment (0–80 μM) on α-tubulin acetylation, MT-associated proteins, and NudC protein respectively in PCa PC-3 and DU-145 cells. (D) The cell cycle distribution as analyzed by flow cytometry. PC-3 cells were treated with fisetin (0–80 μM) for 24 h. Data form a typical experiment performed three times with similar results.

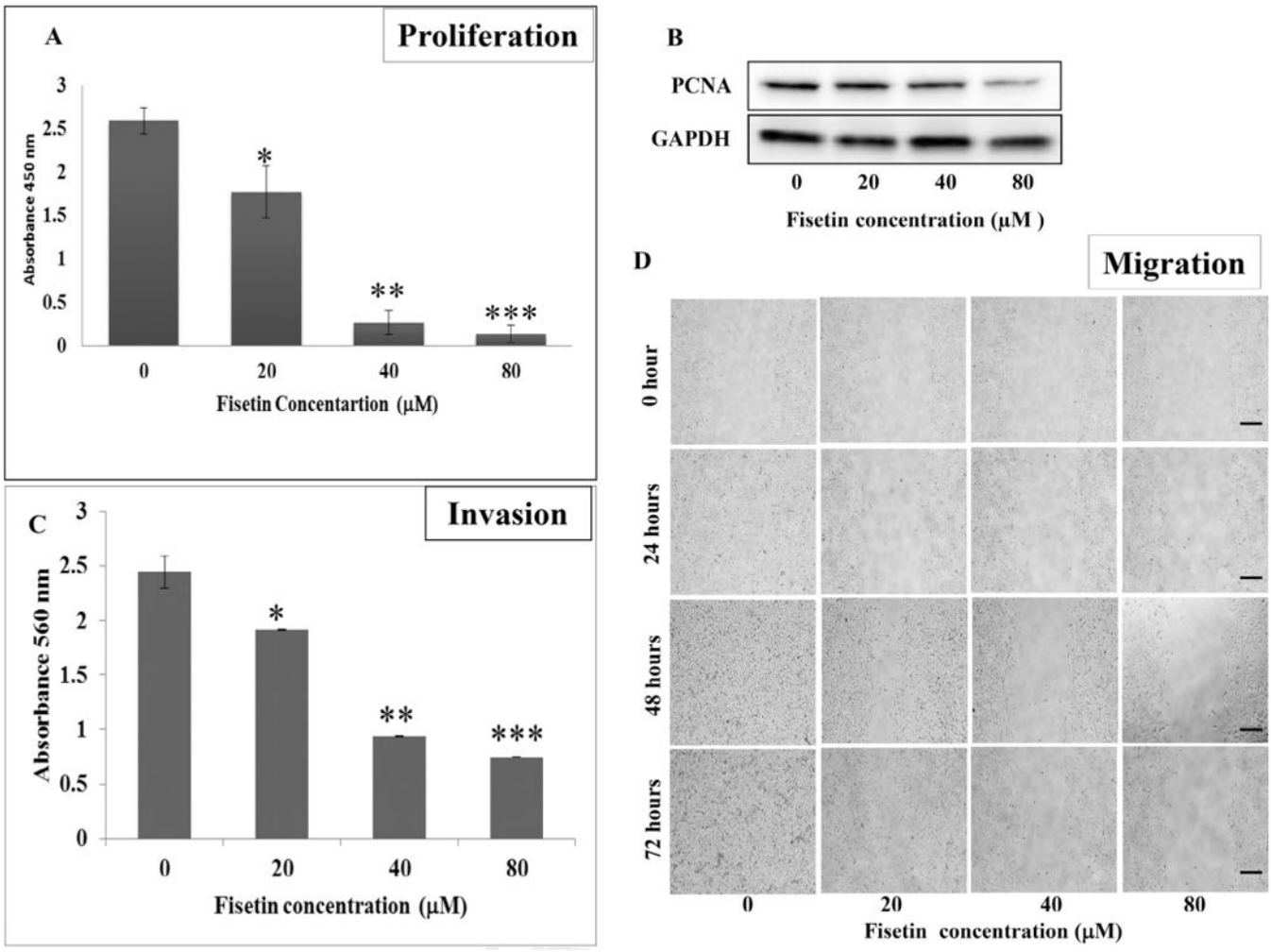

Fig. 6.

Effect of fisetin treatment on PCa cell proliferation, invasion and migration. (A and C) Representative histogram images of PC-3 cells showing proliferation and invasion with or without fisetin treatment at indicated concentrations. Mean cell numbers/field ± SD of experiments performed in triplicate are shown. $^{*}p < 0.05$, $^{**}p < 0.02$, $^{***}p < 0.001$. (B) Representative blots showing the effect of fisetin treatment (0–80 μM) on PCNA in PC-3 cells. (D) PC-3 cells were treated with or without fisetin as indicated. Images were acquired at 0, 24, 48 and 72 h in *in vitro* scratch wound assay. Data shown are representative of three independent experiments for each experiments assay. Scale bar = 60 μm.

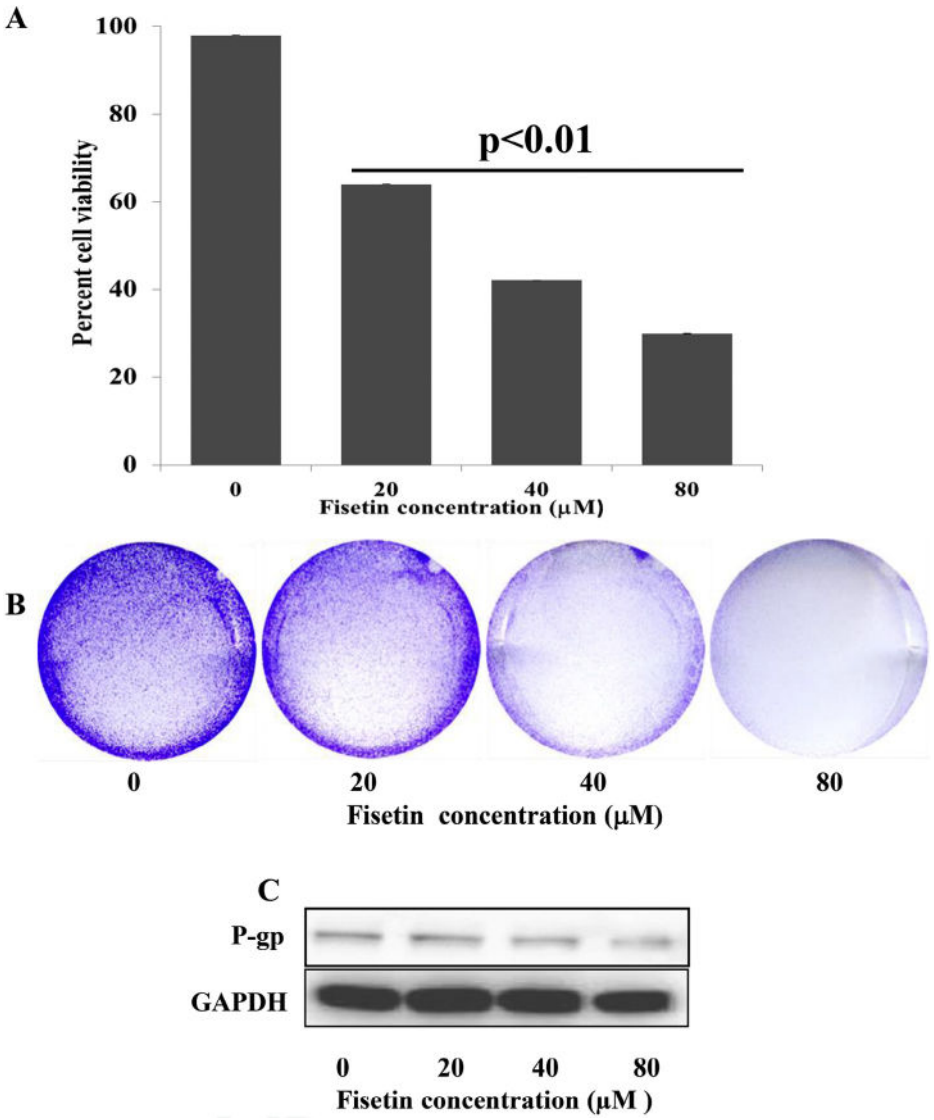

Fig. 7.

Effect of fisetin treatment on cell viability, colony formation and P-gp in NCI/ADR-RES cells. (A) Representative histogram images of NCI/ADR-RES cells showing cell viability assessed by trypan blue exclusion assay with or without fisetin treatment at indicted concentrations. (B) Representative photograph showed the effect of fisetin treatment on NCI/ADR-RES cells growth was investigated by monolayer colony formation assay. (C) Representative blots showing the effect of fisetin treatment (0–80 μM) on P-gp activity. Data shown are representative of three independent experiments for each experiment assay.

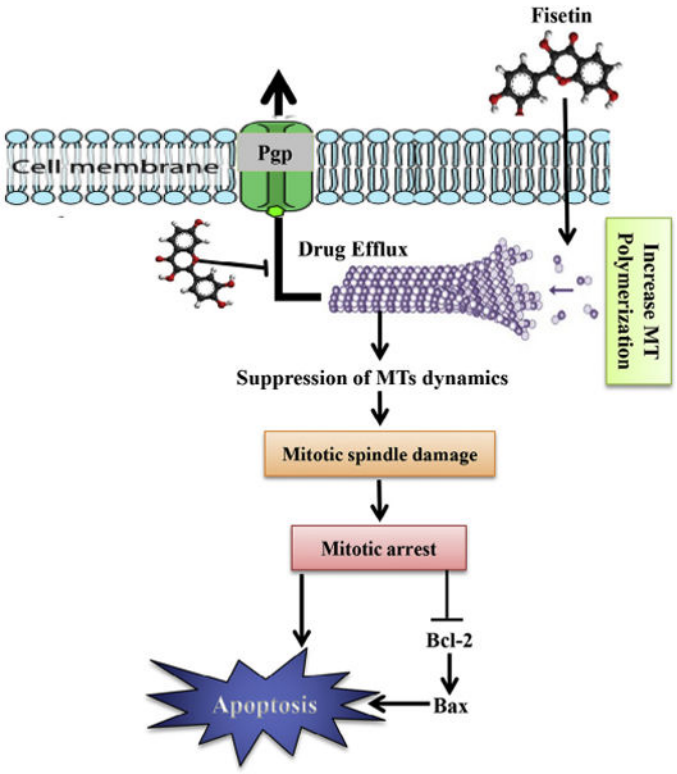

Fig. 8.

Schematic diagram of putative events involved in fisetin induced microtubule stabilization and apoptosis. The interaction of fisetin with β-tubulin stabilize microtubules resulting in disruption of microtubule dynamics that leads to damage to the mitotic spindle. This triggers cell cycle arrest at the G2/M phase subsequently leading to apoptotic cell death. Cancer cells also become resistant to drugs by activating drug efflux pump. Fisetin treatment inhibits drug efflux by downregulating Pg-p.