Anti-cancer activity of a combination of cisplatin and fisetin in embryonal carcinoma cells and xenograft tumors

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Running title: Anti-cancer activity of cisplatin and fisetin combination

Key-words: flavonoid, cisplatin, fisetin, combination chemotherapy, apoptosis, fas, p53

Abbreviations list.
The abbreviations used are: 2-ME, 2-merceptoethanol; Ac-DEVD-AMC, Ac- Asp-Glu-Val-Asp-7-amino-4-methoxy coumarin; Ac-IETD-AFC, Ac-Ile-Glu-Thr-Asp-7-amino-4-trifluoromethyl coumarin; EDTA, Ethylenediaminetetraacetate; ES grade FBS, Embryonic Stem cell grade Fetal Bovine Serum; JC-1, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl carbocyanine iodide; PARP, Poly (ADP-ribose) polymerase; SDS-PAGE, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis ; shRNA, short hairpin RNA; siRNA, small interfering RNA; TUNEL, Terminal deoxynucleotidyl transferase dUTP nick end labelling; VT, Vehicle Treated; Z-DEVD-fmk, acetyl-Asp-Glu-Val-Asp-
fluoromethyl ketone; Z-IETD-fmk, N-benzyloxy carbonyl-Ile-Glu(OMe)-Thr-Asp(OMe)-fluoromethyl ketone; Z-LEHD-fmk, N-benzyloxy carbonyl-Leu-Glu(OMe)-His-Asp(OMe)-fluoromethyl ketone; Z-VAD-fmk, N-benzyloxy carbonyl-Val-Ala-Asp(OMe)-fluoromethyl ketone;

Footnote:

Financial support for research came from funds of the Department of Biotechnology, Govt. of India, numbers BT/03/033/88 and BT/PR/14549/MED/1291/2010 head of accounts 86.3425.02.05.31 and 86.3425.29.04.31. The fund 86.3425.02.05.31 supported doctoral fellowship to Rakshamani Tripathi, and salary support to Sarika Gupta, Avadhesha Surolia and Chandrima Shaha. Tanmoy Sammadder was supported by a Summer Research Fellowship funded by the Indian Academy of Sciences, Bangalore (NII/ACAD/ 3.4/ SUMRES .PROJ. 2008/ACC/08/95).

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Abstract

Use of chemotherapeutic drug cisplatin is limited because of its toxicity. Therefore, efforts continue for the discovery of novel combination therapies with cisplatin to reduce its effective treatment dose. This study evaluates the potential of fisetin, a flavonoid, to increase cisplatin cytotoxicity in human embryonal carcinoma NT2/D1 cells. Addition of fisetin to cisplatin enhanced cisplatin cytotoxicity \textit{in vitro} at four times lower dose than that required by cisplatin monotherapy for similar cytotoxic effects. Cisplatin, fisetin monotherapy and addition of fisetin to cisplatin in a combination increased FasL expression. Cisplatin and fisetin as single agents activated caspases-8 and -3 and caspases-9 and -7 respectively while combination treatment activated all four caspases. Increases in p53 and p21 and decreases in cyclin B1 and survivin occurred, all effects being more exaggerated with the combination. Fisetin, with or without cisplatin, increased expression of proapoptotic protein Bak and induced its mitochondrial oligomerization. Bid truncation and mitochondrial translocation of Bid and p53 was induced by fisetin in the presence or absence of cisplatin. Downregulation of p53 by shRNA during drug treatment decreased p21 levels but caused survivin increase thus reducing cell death. Upstream to p53, inhibition of p38 phosphorylation reduced p53 phosphorylation and cell death. In a NT2/D1 mouse xenograft model, combination therapy was most effective in reducing tumor size. In summary, findings of this study suggest that addition of fisetin to cisplatin activates both the mitochondrial and the cell death receptor pathway and could be a promising regimen for the elimination of embryonal carcinoma cells.
Introduction

Over the last 40 years, incidences of testicular carcinoma have steadily increased becoming the most common cancer of young men [1;2]. A percentage of these carcinomas originating from germ cells either do not respond to treatment or recur post chemotherapy [2;3]. Testicular teratocarcinomas and embryonal carcinoma cells derived from these tumors are extremely sensitive to cisplatin (cis-diamminedichloroplatinum(II)) and its derivatives [4-6], however, use of high doses of cisplatin is limited by the occurrence of various side-effects [7;8]. Combination regimens with cisplatin have been tried with moderate success, however, the search continues for improved formulations [9;10].

Flavonoids, a group of plant secondary metabolites show anti-cancer properties in vitro and in animal models of carcinogenesis of a variety of cancers and a number of them are undergoing clinical trials [11]. Flavonoids genistein, quercetin, silybinin, fisetin are known to augment effects of chemotherapeutic agents [12-14]. A combination of phenoxodiol, an analogue of isoflavone genistein [15] together with carboplatin has entered phase-III clinical trials for the treatment of ovarian cancer (http://www.OVATUREtrial.com). The above reports suggest the importance of exploring and improving flavonoid combinations for cancer treatment. Fisetin (3,3’,4’,7-tetrahydroxyflavone), a flavonoid found in fruits and vegetables inhibits proliferation of multiple types of cancer cells [16-18] through the inhibition of ERK phosphorylation [18], topoisomerase activation [19], induction of PI3K/Akt, JNK signalling [20], caspase-3, -9 and -7 activation, p53 [16] and NF-κB [14] expression. Because of its ability to affect multiple signalling pathways, fisetin is a suitable candidate to serve as a partner for chemotherapeutic agents for the formulation of novel treatment regimens with higher potency.

The specific goals of this study were (i) to determine the anti-cancer efficacy of a combination of cisplatin and fisetin on embryonal carcinoma cells in vitro, (ii) to obtain
mechanistic insights into the mode of action of the combination, (iii) to determine if this combination could affect tumor growth in vivo. The results show that when fisetin is combined with cisplatin, cell death induced is much higher as compared to monotherapeutic treatments with both agents in vitro and in vivo. Also, the effective dose of cisplatin required to induce significant cell death is reduced when combined with fisetin. Mechanistically, multiple death pathways are activated by the combination that generates the amplified effect on cell death.

Materials and Methods

Materials

Cisplatin (cis-diamminedichloro platinum (II)), Fisetin (3,3',4',7-tetrahydroxyflavone, 5-dexoyquercetin), SB 203580, Collagenase type-II DNase I, propidium iodide (PI), custom made oligonucleotides and all other chemicals unless mentioned were obtained from Sigma-Aldrich (St. Louis, MO). Antibodies to caspase-8, caspase-3, FasL (CD95L), Bid, Bak and caspase inhibitors Z-VAD-fmk, Z-IETD-fmk, Z-DEVD-fmk, Z-LEHD-fmk, caspase substrates Ac-IETD-AFC, Ac-IETD-CHO and Matrigel™ were from BD Biosciences (San Jose, CA). Mitochondrial hsp-60, anti-phospho p38 (thr 180, tyr 182) and total p38 antibodies were from StressGen Biotechnologies (Victoria, BC, Canada). Antibodies against cyclin B1, PARP, histone, GAPDH, cytochrome C, caspase-9 and p53 were from Santa Cruz Biotechnology (Santa Cruz, CA) and antibodies against caspase-7, p21 waf1/cip1, survivin, phosphoserine-15-p53 were from Cell Signaling Technology (Danvers, MA). Anti-p53 antibody for immunofluorescence and anti-actin antibodies came from Calbiochem (Darmstadt, Germany), while all secondary antibodies were from Jackson Immuno Research Laboratory (Cambridgeshire, UK). Terminal deoxynucleotidyltransferase-mediated dUTP nick end labelling (TUNEL) kit and CellTiter 96®AQueous non-radioactive Cell Proliferation
Assay kit (MTS assay) were from Promega (Madison, WI). Mitotracker® Red, Hoechst 33342, JC-1 and EnzCheck® caspase-3 assay kit#1 were from Molecular Probes (Eugene, OR). Negative control and p53 siRNAs were purchased from Ambion (Austin, TX) and Dharmaco (Lafayette, CO) respectively. Transpass R2 was from New England Biolabs (Ipswich, MA). G Biosciences (St. Louis, MA) and Biological Industries (Kibbutz Beithaemek, Israel) supplied CBX™ protein estimation kit and Fetal Bovine Serum (FBS) and enhanced chemiluminiscence system respectively. FuGENE-6 was purchased from Roche Diagnostics (Indianapolis, IN).

**Cells**

NT2/D1 cells, a pluripotent cell line (kind gift from Dr. M. Inamdar of JNCASR, Bangalore with consent from Dr. P. Andrews, University of Sheffield, U.K.) derived from a human teratocarcinoma were maintained in DMEM (Dulbecco’s Modified Eagles Medium) with 10% heat inactivated ES grade FBS at 37°C in a humidified atmosphere of 95% air and 5% CO₂. The cell line was tested for the presence or absence of specific surface markers like CD9, CD90, CD133, Nestin, BMI-1 and Oct 4 by flow-cytometry and RT-PCR as a routine to check cell characteristics. The doubling time for the NT2/D1 cells in culture was 13-14 h.

Vehicle, cisplatin and fisetin as monotherapy as well as in combination were added to NT2/D1 *in vitro* cultures at different doses mentioned in the results section. Treatments for xenograft tumors were given as injections daily for a period of 10 days intraperitoneally at doses mentioned in the results section.

**Cell Viability and measurement of mitochondrial membrane potential (MMP)**

For cell viability, CellTiter 96® AQueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI) was used as per manufacturer's instructions. Briefly, treated cells
were incubated with MTS reagent at 37°C (4 h) and absorbance was measured at 470 nm with a Microplate Reader (μQuant, Bio-tek instruments, Winooski, VT). Combination index (CI) was derived from cell death indices achieved with cisplatin (0.5-10 μg/ml) plus fisetin (2–40 μM) at a fixed concentration ratio of 1:4. On the basis of an isobologram generated from the above data for mutually exclusive effects, the CI value was calculated as: combination index (CI) = (D₁/Dₐ₁) + (D₂/Dₐ₂), where (Dₐ₁) and (Dₐ₂) are the concentrations of the single drugs required to induce cell death by 80%, and (D₁) and (D₂) are the drug concentrations in combination treatments which also induce cell death by 80% (isoeffective as compared with single drugs). CI<1, CI=1 and CI>1 indicate synergism, additive effect and antagonism respectively [21]. Cells fixed in 70% ice-cold ethanol and stained with PI were analysed by flow cytometry for detection of membrane permeable cells. MMP was measured using a potentiometric probe JC-1 as described previously [22].

Subcellular fractionation

For subcellular fractionation, cells swelled in hypotonic buffer (10 mM NaCl, 1.5 mM MgCl₂, 10 mM Tris-HCl pH 7.5) were homogenized with a Dounce homogenizer (50 strokes) and resuspended in mitochondrial stabilization buffer (525 mM mannitol, 175 mM sucrose, 1.25 mM Tris-HCl pH 7.5, 2.5 mM EDTA, pH 7.5). The homogenate was centrifuged at 1300 x g to isolate the nuclear fraction followed by centrifugation at 17000 x g in Optima XL-100K (Beckman Coulter, Brea, CA) to isolate the mitochondrial fraction. Post mitochondrial supernatant centrifuged at 100,000 x g provided the cytosolic fraction [23].

Caspase activity assay

Activities of caspases -3 and -8 were assayed according to manufacturer’s instructions using the caspase specific fluorescence peptide substrates Ac-DEVD-AMC and Ac-IETD-
AFC respectively. Fluorescence from free 7-amino-4-methyl coumarin (AMC) was monitored in Fluostar Omega (BMG Labtech, GmBH, Germany) using 340 nm excitation and 485 nm emission and AFC (7-amino-4-trifluoromethyl coumarin) using spectrofluorometer (Perkin-Elmer, Waltham, MA) with excitation wavelength of 400 nm and emission of 450-550 nm.

**SDS-PAGE and Western Blot**

Cells were lysed with lysis buffer (0.125M Tris, 4% SDS, 20% glycerol, and 10% 2-ME). Protein concentrations were determined by the CBX protein assay kit (G-biosciences). Cell lysates (20-50 μg) were electrophoresed in 12% SDS polyacrylamide gels and transferred onto nitrocellulose membranes at room temp. Blocking was done in 5% nonfat dry milk for all non-phospho antibodies used and 5% BSA for all anti-phospho antibodies in 0.05% PBS-Tween 20. The membranes were incubated first with primary antibodies at required dilutions (anti-caspase-8,-9,-3, cyclin B1, cytochrome C and Bak (1:1000), anti-mhsp60, phosphor-p53, phspho-p38, total p38, histone, p21 and survivin (1:2000), anti-p53 (1:4000), FasL, GAPDH and Bid (1:5000) and Actin (1:10000)) in PBS-Tween 20 containing 1% of appropriate blocking reagent and incubated overnight at 4°C. The blots were subsequently incubated with secondary antibody conjugated to horseradish peroxidise at 1:5000-1:20000 dilution in 0.05% PBS-Tween 20 for 1 h at room temp. Protein bands were visualized on X-ray films using the enhanced chemiluminescence system [23].

**Downregulation of p53 using short hairpin (sh) RNA and small interfering (si) RNA**

We obtained pLKO.1-based short hairpin constructs specific for p53 (Addgene plasmid 19119) from the plasmid repository at Addgene [24]. Plasmids containing a short hairpin construct specific for p53 and vector control were transfected together with the
lentiviral packaging (pMDL), envelope (CMV-VSVG), and rev-expressing (RSV-REV) constructs into actively growing HEK293 cells as per protocol provided by Addgene using FuGENE-6 as the transfection reagent. Virus-containing supernatants were collected 48 h after transfection and NT2/D1 cells were infected and selected for puromycin resistance and analyzed at 48 to 72 h after infection.

Cells were transfected with 40 pmol SMART pool Tp53 or negative control siRNA using Transpass R2 transfection reagent as per manufacturer’s instructions (New England Biolabs, Ipswich, MA). Transfection complex formation was initiated by mixing siRNA with transfection reagent prior to incubating them with $10^5$ cells/well/24 well plate for 6 h. Target protein knockdown was assessed 24 h post-transfection by probing extracts of cells on Western blots with anti-p53 antibodies.

### Histology and Immunocytochemistry

Cells were labeled with Mitotracker® Red for specific staining of mitochondria prior to fixation in chilled methanol followed by staining with anti-p53 antibody and secondary antibody conjugated to Alexa-fluor 488 (Jackson Immuno Research Laboratory, Cambridgeshire, UK). An inverted microscope ((TE-2000E) Nikon, Japan) equipped with a RetigaExi camera (Q-imaging, Surrey, BC, Canada) was used to acquire images and coefficient of co-localization was calculated from mask of co-localization using Image-Pro Plus software (Media cybernetics, Silver Spring, MD). For TUNEL labeling, procedure was carried out as described previously [25].

After euthanasia, animals were dissected for removal of tumors and various other organs for fixation in 4% formaldehyde and Bouin’s fluid for TUNEL staining and routine histology respectively. Tissues were processed as per standard protocol [26].
Xenograft tumors

All experimental procedures were performed according to the standards specified by Institutional Animal Ethics Committee of National Institute of Immunology, New Delhi. NT2/D1 cells (10^6) mixed with Matrigel™ were implanted into 6 week old athymic nude mice bearing the nu/nu gene (NIH(s) (nu/nu)) and housed under pathogen-free conditions. The tumor (300-350 mm³) bearing mice were treated with cisplatin (2.5 mg/kg), fisetin (2 mg/kg) and combination of both the drugs where individual dosage was (cisplatin, 1.5 mg/kg + fisetin, 1 mg/kg) intraperitoneally per day for 10 days. Another group of animals were treated with monotherapy of cisplatin (1.5 mg/kg) and fisetin (1 mg/kg). Controls were treated with vehicle only. Xenografted tumor volume was measured using Vernier callipers (major and minor axis) and tumor volume was calculated by the equation: \( L \times \frac{W^2}{2} \) (mm³), where \( L = \text{length} \) and \( W = \text{width} \).

Statistical analysis

Data were analyzed by student’s unpaired \( t \)-test. The values were considered significantly different at \( p < 0.05 \). All experiments were repeated three to five times and data expressed as the mean ± S.E. of several independent experiments.
Results

Cytotoxic activity of cisplatin and fisetin is enhanced when combined together

To determine the cytotoxic effects of cisplatin when given along with fisetin, NT2/D1 cells were exposed to cisplatin and fisetin (Fig. 1A) as monotherapies as well as in combination. Moderate cytotoxic activity with both compounds as monotherapy was evident with a dose dependent reduction in cell number obtained using the MTS assay (dosage range cisplatin, 0.5-100 μg; fisetin, 2-100) (Fig. 1B). A combination of both drugs at a fixed ratio (1:4) over a dosage range, was tested for possible potentiation of cisplatin effects by fisetin (Fig. 1B). These data were used to determine combination index (CI) values through an isobologram [21] calculated as mentioned in the “Materials and Methods”. Combination doses from 2 μg/ml cisplatin and 8 μM of fisetin to 4 μg/ml cisplatin and 16 μM of fisetin fell within the envelope of nearly additive effects (Fig. 1B). A CI value of <1 (0.65) was obtained with 5 μg/ml of cisplatin and 20 μM of fisetin (Fig. 1B) indicating that the interaction between cisplatin and fisetin was synergistic at this dose. This particular combination dose was used for further studies on mechanistic aspects.

Cisplatin, fisetin and the combination induces apoptotic death

We next tested if the cytotoxicity observed was due to cellular apoptosis. Fig. 1C demonstrates a significant increase in sub-G1 cell population representing formation of apoptotic bodies [27] with the combination treatment over a 10 h period. Monotherapy with either cisplatin or fisetin showed approximately 36% lower accumulation of sub-G1 cells suggesting higher rate of apoptosis when fisetin was added to cisplatin. To dissect the mechanism for cell death, cells pretreated with caspase inhibitors were exposed to the drugs to assess if cell death was caspase dependent. Z-VAD-fmk, a pan caspase inhibitor, blocked
cell death in all groups (Fig 1D, c, h, m) suggesting caspase involvement with all treatments. Having established that induction of cell death was caspase dependent, efforts were made to identify specific caspases induced by different treatments with the use of inhibitors specific for particular caspases. Inhibition of caspase-3 only with Z-DEVD-fmk blocked death in cisplatin (f) and the combination treated cells (p) but not in fisetin treated cells (k)(Fig. 1D). In contrast, Z-LEHD-fmk, a caspase-9 inhibitor, was most efficient in reducing cell death with fisetin only (j) and the combination treatment (o) (Fig. 1D). Z-IETD-fmk, the caspase-8 inhibitor could not reduce cell death in fisetin treated cells but was effective in cisplatin (d) and the combination treated cells (n) (Fig. 1D). Further confirmation of specific caspase involvement was determined by monitoring caspase cleavage and activity. Significant cleavage of procaspase-3 as seen on western blots and increased cleavage of fluorescent caspase-3 substrate by lysates from cisplatin and the combination treated cells further confirmed caspase-3 participation in these two treatment groups (Fig. 2A). Procaspase-9 proteolysis was most prominent with fisetin and the combination treatment at 8 h (Fig. 2B), which is in concurrence with the ability of caspase-9 inhibitor to block cell death in these two groups shown earlier in Fig. 1D. Proteolysis of procaspase-8 to the active 23 kDa fragment was detected in cell lysates from only cisplatin and the combination treatment (Fig. 2C). Fisetin could induce only partial proteolysis of procaspase-8 to the 40/36 kDa form while the combination could generate both the 40/36 kDa and the 23 kDa form (Fig. 2C). These observations were supported by higher caspase-8 activity obtained with cell lysates from cisplatin and the combination treated cells, using Ac-IETD-CHO as a caspase-8 substrate (Fig. 2C). These data suggest that the increase in antiproliferative activity observed with the combination of cisplatin and fisetin is attributable at least in part to significant cellular apoptosis mediated by caspases.
The role of FasL in the activation of cell death receptor pathway is well established [28]. Increased expression of FasL (Fig. 2D) verified activation of the death receptor pathway in all groups. Since complete proteolysis of procaspase-8 occurred with cisplatin but not with fisetin, signals downstream to caspase-8 proteolysis were investigated. The partial cleavage of caspase-8 to 40/36 kDa fragment in combination and fisetin treated cell lysates prompted us to check for the truncation of Bid, as Bid truncation is known to be induced by incompletely cleaved caspase-8 [29]. Truncated Bid was visible in cell lysates from fisetin and the combination treated group with more complete cleavage occurring upon combination treatment (Fig. 2D). Procaspase-7, an executioner caspase that can be activated by caspase-9 [28] was cleaved at 6 h to its active form in the combination group and at 10 h in the fisetin treated group (Fig. 2D) suggesting faster activation with the combination. The lack of visibility of cleavage product with the combination treatment at 10 h could be due to death of a large number of cells by then and the procaspase-7 band visible probably originates from remaining surviving cells. The cleavage of caspase-7 was caspase-9 dependent as caspase-9 inhibitor Z-LEHD-fmk blocked caspase-7 cleavage when present during drug treatment (Fig. S1). In summary, participation of the death receptor pathway in execution of apoptosis was evident in all groups with cisplatin and fisetin using different initiator and executioner caspases. Addition of fisetin to cisplatin induced caspases-8,-3,-9 and -7 in tandem, possibly inducing amplification of effects with the combination.

Cisplatin and fisetin combination induces p53 mediated death pathway

The tumor suppressor protein p53 coordinates a complex network of cellular proteins evolved to protect cells from malignant transformation and can play a proapoptotic role [30]. Unlike many other malignant cells, NT2/D1 expresses an active p53 [31]. The p53 expression increased with all 3 treatments but this increase was most significant with fisetin and the
combination treatment (Fig. 3A). The downstream transcriptional target for p53, p21 protein levels also increased in a similar fashion as p53 (Fig. 3A). Cyclin B1, a regulatory protein involved in mitosis [32] was downregulated to a greater degree in the combination treated cell lysates as compared to fisetin while cisplatin did not induce any significant change (Fig. 3A). Survivin, an inhibitor of IAP (inhibitor of apoptosis protein) [32] proteins was significantly downregulated with the combination treatment as compared to monotherpeutic administrations (Fig. 3A). Subcellular localization of p53 is indicative of its function. Investigations into the p53 subcellular localization demonstrated heavy nuclear concentration of the protein with the combination treatment but cisplatin and fisetin monotherapy did not induce any significant change (Fig. 3B, Fig. S2). Mitochondrial localization of p53 where it can act as ‘BH3 only protein’ is known [33]. With our studies it is evident that fisetin treatment with or without cisplatin induced higher p53 concentration in the mitochondria (Fig. 3B, Fig. S3, S4, S5 vehicle control). In contrast to fisetin, cisplatin treatment did not induce any mitochondrial accumulation of p53 (Fig. 3B, Fig. S2). Post translational modifications like phosphorylation induce protein activity [34]. Phosphorylation of p53 is predicted to be essential for its stabilization, activation of target genes and induction of apoptosis [35]. Phosphorylation at Ser-15 is linked to cellular apoptosis [35] and in NT2/D1 cells, phosphorylation occurred at Ser-15 position (Fig 3C) with the combination treatment suggesting proapoptotic role of p53 in NT2/D1 apoptosis.

The changes in p53 described above suggested a major role of the molecule in mediating the effects of cisplatin and fisetin on cell death. This prompted us to investigate cellular response in the absence of p53. A stable cell-line was generated through the expression of p53 shRNA, where cellular expression of p53 was significantly lower as compared to non-transfected cells (Fig. 3D). As a consequence of p53 downregulation, a prominent decrease in p21 levels occurred in all 3 groups with concomitant upregulation of
survivin (Fig. 3D). As shown in Fig. 3D, the treatments could induce lower cell death under p53 knockdown conditions as compared to cells with normal constitutive p53 expression. These biochemical alterations are reflected in morphological changes. Our studies demonstrate a distinct difference in cellular morphology between the drug treated cells in the vector controls versus the p53 knockdown cells (Fig. 3D, cell morphology). Detachment of cells from the substratum and rounding up were the prominent features of treated cells transfected with vector only, while cells under p53 knockdown conditions show adherent cells with clear spread out morphology and lesser detachment from the substratum. Knockdown of p53 using siRNA showed similar results with the different treatments (Fig. S6).

Mitochondria is involved in fisetin mediated cell death

The truncation of Bid suggested mitochondrial involvement in drug induced cellular apoptosis and one of the established methods to assess mitochondrial function is measuring the loss of mitochondrial membrane potential [36]. We observed a significant loss of potential in the combination as well as the fisetin treated cells (Fig. 4A). Mitochondrial potential fall results in cytochrome c release to the cytosol [36]. Cytochrome c release was observed with both fisetin and the combination treatment (Fig 4B) suggesting mitochondrial involvement these two treatment models. Other than Bid translocation to the mitochondria, proapoptotic proteins like Bak could be involved in the release of cytochrome c as well [36]. Significant upregulation of Bak along with its oligimerization in the mitochondria was observed most prominently with the combination (Fig 4C). PARP cleavage [28] which is a hallmark of caspase-3 and -7 activation was visible with all 3 groups with the combination inducing significant cleavage by 10 h (Fig. 4D).
Cisplatin and fisetin combination increases phosphorylation of p38

Prior knowledge that p38 regulates events upstream to p53 [37], prompted us to investigate possible changes in p38. No change was observed in total expression levels of p38 but increased phosphorylation (Thr 180/Tyr182) occurred with all treatments (Fig. 5A). Since phosphorylation regulates activity of proteins, phosphorylation inhibitor for p38, SB203580 [37] was used to inhibit p38 phosphorylation (Fig. 5A). As a consequence of p38 phosphorylation inhibition, there was a reduction in p53 phosphorylation as well (Fig. 5B). Biological parameters like mitochondrial potential fall as a consequence of drug treatments was inhibited by SB203580 (Fig. 5C). Changes in potential were markedly induced by cisplatin and fisetin together that could be inhibited by SB203580. This inhibition translated into the rescue of cells from death in all groups being most significant with the combination. These observations suggested involvement of p38 in p53 mediated effects (Fig. 5D).

Combination of cisplatin and fisetin inhibits growth of xenograft tumor in mice

To examine if our studies in vitro were reproducible in a model of NT2/D1 xenograft, tumors were induced in athymic nude mice. Monotherapeutic doses of cisplatin (2.5 mg/kg/day) and fisetin (2 mg/kg/day) showed significant regression of tumors (Fig. 6A and B; Fig. S7). Addition of fisetin to cisplatin at the same dose as above was toxic and the mice did not survive beyond 5 days of treatment. However, addition of a lower dose of fisetin (1 mg/kg/day) to cisplatin (1.5 mg/kg/day) was not cytotoxic but showed significant reduction of tumor size over a period of 10 days (Fig. 6A and B; Fig. S7). The extent of tumor regression achieved with this treatment was significantly more than the monotherapeutic treatments (Fig. 6A and B). There was no significant reduction in body weight post treatment except in the control or the cisplatin treated group (Fig. 6C). To determine the extent of cellular apoptosis within the tumors, tissue sections were prepared for TUNEL staining. As
shown in Fig. 6D, TUNEL positive apoptotic cells were detectable in tumor sections from monotherapeutic treatments of cisplatin and fisetin but not in vehicle treated tumors (Fig. 6D). However, a large population of TUNEL positive cells as shown by cells with brown nuclear deposits, was substantially higher in the combination group on day 5 of treatment when cisplatin was administered along with fisetin (Fig. 6D). Sections from 5 day old tumors are presented in the figures as these tumors were of reasonable size for obtaining a large enough cell population (Fig. 6D).

For checking the cytotoxic potential of the different treatment schedules, histological sections from the kidney of treated animals were stained for apoptotic cells. Very few TUNEL positive cells were visible in the kidney of animals receiving monotherapeutic treatments with cisplatin (2.5 mg/kg/day), fisetin (2 mg/kg/day) and the combination (cisplatin, 1.5 mg/kg/day + fisetin, 1 mg/kg/day) (Fig. S8). However, a higher number of TUNEL positive cells were detected in the kidney cortex of animals treated with the combination of both drugs at a higher dose (cisplatin, 2.5 mg/kg/day + fisetin, 2 mg/kg/day) on day 4 of treatment (Fig. S8). Cellular morphology did not show any marked change in liver, spleen and kidney of the monotherapies and the combination group (cisplatin, 1.5 mg/kg/day + fisetin, 1 mg/kg/day) (Fig. S9A-C).
Discussion

NT2/D1 teratocarcinoma cells *in vitro* and in a xenograft model were used to investigate a potential strategy for increasing the effectiveness of cisplatin in killing teratocarcinoma cells by combining the flavonoid fisetin with cisplatin. Cisplatin is one of the most widely used chemotherapeutic drug [38;39] and fisetin is a flavonoid known to eliminate cancer cells [14]. Recently, fisetin’s role as a potentiation factor in killing cancer cells has been shown by its augmentative actions on the induction of cell death by radiotherapy in colorectal cancer cells and with cisplatin in Burkitt’s lymphoma cells [14;40]. However, the mechanism by which fisetin contributes to the increase in cytotoxic activities of such treatments is not known. Our findings from *in vitro* and *in vivo* studies support the hypothesis that combining fisetin with cisplatin potentiates cisplatin’s ability to induce apoptosis of NT2/D1 cells and provoke NT2/D1 xenograft tumor regression. The clinical implications of these laboratory findings suggest that: (a) for a fixed dose of cisplatin, addition of fisetin may improve antitumor responses; (b) cisplatin may be given at a reduced and potentially less toxic dose in combination with fisetin; (c) components of the involved apoptotic pathways identified could be manipulated to achieve further improvement in treatment modalities.

Our observations on cisplatin induced death of NT2/D1 cells both *in vitro* and *in vivo* is consistent with the ability of testicular teratocarcinomas to respond favorably to treatment with cisplatin [41;42]. Sensitivity of embryonal carcinoma cells to fisetin only treatment observed in these studies add to observations on fisetin induced death of prostate, pancreatic, colon and hepatocellular carcinoma cells [16;17;43;44]. Adding fisetin with cisplatin as a combination to NT2/D1 cells, resulted in significantly greater cell death with a CI value of 0.65 suggesting synergism [45;46], clearly establishing fisetin’s ability to potentiate cisplatin effects. Interestingly, in prostate cancer PC3 cells, autophagic death is induced by fisetin...
through suppression of mTOR [47], an observation not reproduced in NT2/D1 cells at the dose used (data not shown).

The observations of increased expression of FasL and procaspase-8 proteolysis [28] with all three treatments clearly indicated activation of caspase dependent death pathway through death receptor signaling. However, downstream to procaspase-8 proteolysis, alternate signaling pathways were engaged by cisplatin and fisetin monotherapy because unlike cisplatin, fisetin induced only partial proteolysis of procaspase-8 that is favorably associated with Bid truncation [29]. This was confirmed by clear Bid truncation with both fisetin monotherapy and the combination suggesting activation of mitochondrial death pathway in these two treatment models [36]. Proapoptotic proteins of the Bcl-2 family induce mitochondrial disruption in terms of loss of mitochondrial potential and cytochrome c release [36]. In our experiments, largely, two proapoptotic mitochondrial proteins, Bid and Bak were involved in the activation of the mitochondrial death pathway with fisetin and the combination treatment resulting in signal amplification. Interestingly, fisetin by itself elicits similar events in human colon and prostate cancer cells [16;43]. The concomitant translocation of p53 to the mitochondria in two groups where Bid and Bak were active suggested that p53 could act as a proapoptotic ‘BH3 only’ protein because Bak is known to be ‘freed’ by p53 on the mitochondrial membrane to be able to form oligomers [30]. The p53 protein is a tumor suppressor which acts as a checkpoint in cell cycle either preventing or initiating programmed cell death [48] and is mutated in many forms of cancer [48]. However, in teratocarcinoma cells, this rate of mutation is very low [41] and therefore, p53 has an important role as observed in this study.

In NT2/D1 cells, fisetin primarily used caspase-7 as executioner caspase instead of caspase-3, which could potentially be activated through the mitochondrial death pathway as
in hepatocellular carcinoma where caspase-3 is activated by fisetin directly [17]. Direct
activation of caspase-3 by cisplatin without mitochondrial involvement is in contrast to
observations in ovarian cancer cells [46] HEp-2 cells [49] and osteosarcoma cells [50] where
cisplatin is reported to induce death through the mitochondrial pathway. However, direct
activation of caspase-3 by cisplatin in other cell types is well known [31]. Therefore, apart
from the disparity in the use of initiator caspases by cisplatin and fisetin, our studies
demonstrate the use of distinct executioner caspases as well. Fisetin treatment primarily
induced caspase-7 activation through procaspase-9 which is similar to observations with HT-
116 colon cancer cells, however, in HT-116 cells caspase-3 is also activated by fisetin [16]
unlike NT2/D1 cells. Clearly, in the combination, the addition of fisetin to cisplatin induced
the use of multiple caspases like caspases-8,-9,-3 and -7 thus amplifying the death inducing
effects.

The differential changes in p53 observed in NT2/D1 cells are similar to effects as seen
in SK-HEP-1 hepatocellular and HT-116 colon carcinoma cells [16;17]. In addition to
mitochondrial translocation, a prominent translocation of p53 to the nucleus [51] suggested
enhanced transcriptional activity possibly resulting in the upregulation of p21, a cyclin
dependent kinase inhibitor. Previously published results show that p21 regulates expression
of survivin, a member of inhibitor to apoptosis protein family [32] in multiple cell types
including hepatocellular carcinoma HepG2 cells [52]. Survivin downregulation during drug
treatment is in agreement in the increased cell death observed. Deregulation of cyclin B1,
which could act as a caspase inhibitor and is connected to neoplastic transformation, is
suggested to be an attractive strategy for antiproliferative therapy [53]. As seen with survivin,
the reduced expression of cyclin B1 was concurrent with increased cell death observed. [37] .
Inhibition of p38 phosphorylation resulted in a decrease of p53 phosphorylation suggesting
p38 mediated regulation of p53 activity. In addition, inhibition of p38 phosphorylation also
led to the rescue of mitochondrial potential and cell death suggesting the connectivity between p38, p53 phosphorylation and cell survival. Our observations of p53 phosphorylation at Ser-15 [35] and ensuing decrease in cell death following inhibition of this phosphorylation reiterate the role of p53 as a proapoptotic modulator.

In agreement with the in vitro data, the NT2/D1 xenograft model showed more efficient tumor regression with the combination therapy as compared to the monotherapies. This was consistent with histological observations where the number of TUNEL positive cells [54] in tumors treated with the combination far exceeded cells observed with only cisplatin or fisetin treatment. At higher doses of the combination, however, toxicity was detected. Histopathology of the kidney showed no significant changes at the lower dose.

Interpretation of these results in terms of mechanistic implications suggest cooperation of multiple pathways in eliciting death signals through cross-talk between p53 and proapoptotic Bid and Bak with activation of upstream sensors like p38 when fisetin was administered with cisplatin. The recruitment of dual death pathways using multiple caspases adds an additional armamentarium for treatment, with possibilities of enhancement of effect through modulation of individual pathway components. Therefore, this study highlights a new possibility of using a combination therapy of cisplatin with a flavonoid fisetin for elimination of stem teratocarcinoma.

Acknowledgements

Technical assistance of Mr. Neelaram is appreciated. NT2/D1 cells were obtained from the Jawaharlal Nehru Center for Advanced Scientific Research, Bangalore, India (courtesy Dr. M. Inamdar with permission from Dr. P. Andrews, Department of Biomedical Science, University of Sheffield, U.K.). Dr. Bob Weinberg of Whitehead Institute for Biomedical
Research, MA, is acknowledged for providing shp53 pLKO.1 puro plasmid through Addgene. Funding from Department of Biotechnology, New Delhi towards ‘Centre for Molecular Medicine’ is acknowledged.
Reference List


Figure Legends

Figure 1. Effect of cisplatin and fisetin on NT2/D1 cell viability. A. Chemical structures of cisplatin and fisetin. B. Cell viability in response to cisplatin and fisetin exposure at increasing doses. The fixed ratio graph shows cytotoxicity of various doses of cisplatin and fisetin combination at a fixed ratio of 1:4. The isobologram generated based on the fixed ratio graph shows the combination of 5 μg/ml cisplatin and 20 μM fisetin (red arrow) as synergistic with a combination index of 0.65. Data are ± SE of 3 experiments. C. Measurement of sub-G1 cell population over a period of 10 h post treatment with fisetin, cisplatin and a combination of fisetin + cisplatin. Data are ± SE of 3 experiments. D. Bar graphs show the percentage of PI positive cells in various treatment groups in the presence or absence of Z-VAD-fmk, pan caspase inhibitor; Z-IETD-fmk, caspase-8 inhibitor; Z-LEHD-fmk, caspase-9 inhibitor and Z-DEVD-fmk, caspase-3 and -7 inhibitor. Data represent the mean percentage of PI positive cells ± SE (n=3). Cis, cisplatin; Fis, fisetin, Cis+Fis, cisplatin + fisetin, Anta., antagonistic; Add., Additive; Syn., synergistic.

Figure 2. Caspase activity during drug treatments. A. Western blot showing the processing of procaspase-3 at 10 h with cisplatin, fisetin and cisplatin + fisetin treatment. Actin blot shows the loading control. Caspase-3 activity plot demonstrates measurement of caspase-3 activity using Ac-DEVD-AMC as substrate with cell lysates collected at 8 h post treatment. Data represent ± SE (n=3). Pro c-3, procaspase-3; casp-3, caspase-3. B. Western blot showing the processing of procaspase-9 at 8 h. Bar graph represents densitometric measurements of procaspase-9 processing on the Western blot. Data are normalized with actin immunoreactivity used as loading controls. Pro c-9, procaspase-9. Data are mean ± SE, n=3. C. Western blot of cell extracts from cisplatin, fisetin and the combination of cisplatin and fisetin treated cells probed with anti-caspase-8 antibody showing cleavage of procaspase-
8 to 46/36 kDa and 23 kDa fragments. Note that fisetin treated cells shows proteolysis of procaspase-8 to 46/36 kDa but not to 23 kDa active fragments. Measurement of caspase-8 activity using Ac-IETD-AFC as substrate shows caspase-8 activity obtained with cell lysates from different treatment groups at 8 h after treatment. Data are representative of a minimum of 3 experiments. Pro c-8, procaspase-8; C, cisplatin; F, fisetin; C+F, cisplatin + fisetin; C+F+I, cisplatin + fisetin + caspase inhibitor. D. Western blots of cell extracts probed with anti-FasL antibody shows increase of FasL expression as compared to vehicle treated controls in all 3 groups of treatment at 6 and 8 h. Bars show relative expression of FasL measured by densitometry and normalized to actin immunoreactivity used as loading controls. Western blots of cell extracts probed with anti-Bid antibody showing the cleavage of Bid with different treatments. Immunoblot analysis for caspase-7 at 6 h and 10 h show cleavage of caspase-7 to its active form. Data are mean ± SE, n=3. Pro c-7, procaspase-7. Cis, cisplatin; Fis, fisetin, Cis+Fis, cisplatin + fisetin.

Figure 3. Changes in cell cycle regulatory molecules. A. Western blots of cell cycle regulatory proteins p21, p53, cyclin B1 and survivin at 4 and 6 h post drug treatment probed with respective antiserum. Actin was used as a loading control. VT, vehicle control. Data are representative of at least 3 experiments. B. Western blots of subcellular fractions of treated and untreated cells probed with anti-p53 antibody. VT, vehicle control; m, mitochondria; n, nuclei; c, cytosol. C. Western blot shows an increase in p53 phosphorylation as well as total p53 expression after treatment with drugs. Densitometric measurements represent relative p53 phosphorylation as normalized against total p53 levels. Data are mean ± SE, n=3. D. Western blots of extracts of NT2/D1 cells with vector and shRNA transfection probed with anti-p53, p21 and survivin antibodies showing relative levels of p53, p21 and survivin. Bar graph shows cell death percentages with different treatments of cells with or without shRNA.
mediated knockdown. Data are mean ± SE, n=3. Microphotographs show cellular
morphology after treatment with cisplatin, fisetin and the combination in vector control and
p53 knocked down cells. Cis, cisplatin; Fis, fisetin, Cis+Fis, cisplatin + fisetin.

**Figure 4.** Changes associated with mitochondria and PARP cleavage. A. Flow
cytometric analysis of treated and untreated cells stained with potentiometric probe JC-1 for
mitochondrial potential measurement. Cis, cisplatin; fis, fisetin; cis + fis, cisplatin+fisetin.
The upper quadrant and lower quadrant shows cells stained with JC-1, red staining (FL-2)
indicating cells with higher mitochondrial potential and green staining (FL-1) indicating cells
with lower mitochondrial potential. Data are representative of 4 experiments. B . Western
blots of subcellular fractions of treated and untreated cells probed with anti-cytochrome c
antibody showing cytochrome c content in the cytosol and the mitochondria. VT, vehicle
control; m, mitochondria c, cytosol. Antibody against GAPDH were used for the fraction
purity. Data are representative of 3-4 experiments. C. Western blots showing expression and
oligomerization of Bak in whole cell and mitochondrial lysates respectively. Relative Bak
eexpression is normalized against actin used as loading control. Data are representative of 3
experiments. Arrows on oligomerization blot indicate oligomerized products. Cytochrome c
levels in the mitochondria are shown below. D. Western blots probed with anti-PARP
antibody shows PARP cleavage with cisplatin, fisetin and cisplatin + fisetin. Cis, cisplatin;
Fis, fisetin, Cis+Fis, cisplatin + fisetin. Data are representative of 3 experiments.

**Figure 5.** Phosphorylation of p38. A. Western blots of treated cell lysates showing
inhibition of p-38 phosphorylation with SB 203580. Relative phospho-p38 shown is a value
normalized against total p38. Actin was used as a loading control. Data are mean ± SE, n=3.
B. Western blots of total cell lysates probed for p53 phosphorylation (ser-15) shows
inhibition of combination induced phosphorylation by SB 203580. Relative p53 expression are
values normalized against actin and relative p53 phosphorylation are values normalized
against total p53. Data are mean ± SE, n=3. C. Figure shows recovery of mitochondrial
potential fall in the presence of SB 203580 primarily with the fisetin and the combination
treatment. Data are representative of at least 4 experiments. D. Bar graph shows cell death
induced by the various treatments in the presence or absence of p-38 phosphorylation
inhibitor SB 203580. Data are mean ± SE, n=3.

Figure 6. NT2/D1 tumor regression in a mouse xenograft model. A. Figure shows
reduction in tumor size after treatment with cisplatin, fisetin and cisplatin + fisetin
combination after 5 and 10 days of continuous treatment. Tumor size demonstrates the size of
the dissected out tumors. B. Graph showing tumor volume changes within 10 days after
initiation of treatment (marked by arrow). Number of animals per group = 5. Data are mean ±
SE, n=4. VT, vehicle control; Cis, cisplatin; Fis, fisetin, Cis+Fis, cisplatin + fisetin. C.
Average body weights of the mice during tumor development and treatment. Data are mean ±
SE, n=4. D. Representative sections from the tumor xenografts dissected out from treated
mice stained for TUNEL showing cells with fragmented DNA in treatments with cisplatin,
fisetin, a combination of cisplatin and fisetin and vehicle treated control tumor. Note the large
number of TUNEL positive cells with brown deposit when cisplatin was added to fisetin. Cis,
cisplatin; Fis, fisetin, Cis+Fis, cisplatin + fisetin.
Fig. 1A-D

(A) Graphical representation of chemical structures with labels Cis and Fis.

(B) Dose response graph showing cell number (% of Control) against dose for Cis and Fis.

(C) Fixed ratio graph showing cell number (% of Control) against Cis (μg/ml) and Fis (μM).

(D) Isobologram showing combination index (CI) and fraction affected.

Author Manuscript Published OnlineFirst on January 7, 2011; DOI: 10.1158/1535-7163.MCT-10-0606
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.
Fig. 3A-D

A

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C

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p<0.01

p<0.05

% PI positive cells

B

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p<0.05

Relative p53 Ser-15 phosphorylation

% PI positive cells

wt NT2/D1

shp53 NT2/D1

p<0.01

p<0.05

p<0.05

p<0.05

p<0.05

p<0.05

p<0.05

p<0.05

p<0.05

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Cell morphology

12h

Vector Control

p53 knock down cells

Vehicle

Cis

Fis

Cis + Fis
**Fig. 4A-D**

A) Flow cytometry analysis showing the percentage of FL1-H and FL2-H for Vehicle, Cis, Fis, and Cis + Fis treatments.

B) Western blot analysis showing the expression of Cyt C and GAPDH after 4 hours of treatment with Cis, Fis, or Cis + Fis.

C) Relative BAK expression and BAK oligomerization after 6 hours of treatment with Cis, Fis, or Cis + Fis. The graph shows a significant increase in BAK expression with p<0.05.

D) Western blot analysis of PARP cleavage and Actin expression for Cis, Fis, and Cis + Fis treatments at various time points (0, 4, 6, 8, 10, 12 hours).
Fig. 5A-D
Fig. 6A-C

A

5 Days after treatment

10 Days after treatment

Tumor size

0.9% Saline
Cisplatin 2.5mg/kg/day
Fisetin 2mg/kg/day
Cisplatin+Fisetin 1.5mg/kg/day + 1 mg/kg/day

B

C

Tumor Volume (mm³)

Average Body Weight (gm)

VT
Cis
Fis
Cis + Fis (low)
Cis + Fis (high)

Time (days)

0 7 11 15 20 22 23 25 27 29 30

0 200 400 600 800 1000 1200

0 27 28 29 30

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Fig. 6D

Vehicle Treated

Cis

Fis

Cis + Fis
Molecular Cancer Therapeutics

Anti-cancer activity of a combination of cisplatin and fisetin in embryonal carcinoma cells and xenograft tumors

Rakshamani Tripathi, Tanmoy Samadder, Sarika Gupta, et al.

Mol Cancer Ther Published OnlineFirst January 7, 2011.

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