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Improved antiangiogenic and antitumour activity of the combination of the natural flavonoid fisetin and cyclophosphamide in Lewis lung carcinoma bearing mice

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ABSTRACT

Purpose: The natural flavonoid fisetin was recently identified as a lead compound that stabilizes endothelial cell microtubules. In this study we investigated the antiproliferative and antiangiogenic properties of fisetin *in vitro* and *in vivo*.

Methods: Fisetin cytotoxicity was evaluated using Lewis lung carcinoma cells (LLC), endothelial cells and NIH 3T3 cells. Endothelial cell (EC) migration and capillary-like structure formation were evaluated using EAhy 926 cells. *In vivo* tumour growth inhibition studies were performed using LLC bearing mice treated with fisetin and/or cyclophosphamide (CPA).

Results: The fisetin IC₅₀ was 59 µM for LLC and 77 µM for EC cells, compared to 210 µM for normal NIH 3T3 cells (24 h). Fisetin inhibited EC migration and capillary-like structure formation at non-cytotoxic concentrations (22-44 µM). In mice, fisetin inhibited angiogenesis assessed using the Matrigel plug assay. In LLC bearing mice, fisetin produced a 67% tumour growth inhibition (223 mg/kg, intraperitoneal), similar to the 66% produced by low dose CPA (30 mg/kg, subcutaneous). When fisetin and CPA were combined, however, a marked improvement in antitumour activity was observed (92% tumour growth inhibition), with low systemic toxicity. Tumour histology showed decreased microvessel density with either fisetin or CPA alone, and a dramatic decrease after the fisetin/CPA combination.

Conclusions: We have shown that fisetin not only displays *in vitro* and *in vivo* antiangiogenic properties, but that it can also markedly improve the *in vivo* antitumour effect of CPA. We propose that this drug combination associating a non-toxic dietary flavonoid with a cytotoxic agent could advantageously be used in the treatment of solid tumours.

Keywords: flavonoid, fisetin, cyclophosphamide, Lewis lung carcinoma, EA-hy 926
endothelial cells, angiogenesis, cytotoxicity, antitumour activity

Background

Tumour vasculature is an attractive target for cancer therapy because a single vessel provides oxygen and nutrients to numerous tumour cells and is the main route for metastatic dissemination of cancer cells (reviewed in [1]). Tumour angiogenesis is the result of an imbalance between pro-angiogenic factors, e.g., vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), and endogenous antiangiogenic factors, such as angiostatin and endostatin [2-4]. Tumour vasculature can be targeted at the angiogenesis level to prevent the formation of new vessels using antiangiogenic agents, or at the vascular level using vascular disrupting agents on already formed vessels [5,6]. The anti-angiogenesis approach has already proven its clinical effectiveness in colon, breast, and non-small-cell lung cancer using VEGF antibody in combination with cytotoxic drugs [7-9].

Several phytochemicals, or compounds derived from edible plants, have been linked to the chemoprevention of cancer [10]. Among these compounds, the natural flavonoids have been shown to display pharmacological properties of interest in the prevention and treatment of cancer, as cytotoxic and/or as antiangiogenic agents [11-14].

In a program aimed at finding novel antiangiogenic agents, we recently identified the natural flavonoid fisetin (3,3',4',7-tetrahydroxyflavone) as an interesting lead that can stabilize endothelial cells *in vitro* at non cytotoxic concentrations (Figure 1) [15]. Fisetin is present in several fruits, vegetables, nuts and wine [16,17], and displays a variety of biological effects including antioxidant, anti-inflammatory [18,19], anti-carcinogenic and *in vitro* angiogenesis [20]. Fisetin has already been shown to be cytotoxic to various human cancer cell lines including leukaemia (HL60) [21], breast (MCF7) [20], colon (HT29) [22], liver (SK-HEP-1,

Caco-2) [22,23], neuroblastoma (SHEP, WAC-2) [20], prostate (LNCaP, PC3) [24], and also to several endothelial cells [20]. Fisetin has been shown to inhibit several molecular targets, including cyclin-dependent kinases [25-27], DNA topoisomerases I and II [28,29], urokinase [30], actin [31], and androgen receptor signalling [32]. It has also recently been found that fisetin induces a forced exit from mitosis by targeting the mitotic spindle checkpoint involving the inhibition of Aurora B activities required for the maintenance of normal spindle checkpoint signalling [33].

In the present study, we further tested fisetin's *in vitro* antiangiogenic action and evaluated its *in vivo* antitumour activity in Lewis lung carcinoma bearing mice. We report here that fisetin displays anti-angiogenic properties *in vitro* as well as *in vivo* inhibition of Lewis lung carcinoma tumour growth involving an anti-angiogenic mechanism. In addition, when fisetin was combined with low dose cyclophosphamide, a remarkable improvement in antitumour activity involving an anti-angiogenic mechanism of action was observed. We propose that this relatively non toxic drug combination using a dietary phytochemical with low-dose cyclophosphamide could advantageously be used in the treatment of solid tumours.

Methods

Chemicals

Fisetin (3,3',4',7-tetrahydroxyflavone) and cyclophosphamide were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). Fisetin stock solution was prepared in dimethylsulfoxide (DMSO) and stored at 4°C in the dark. Cyclophosphamide was dissolved in sterile water.

Cell viability

The murine Lewis lung carcinoma (LLC) cell line, the NIH 3T3 murine fibroblast cell line and the EAhy 926 endothelial cell line (an immortalized human umbilical vein endothelial cell line [34]) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 2 mM L-glutamine, 10% foetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin (37°C, 5% CO₂). Exponentially growing cells were plated onto 96-well plates at 5000 cells per well in 200 µl. After 24 hours, cells were exposed to fisetin at the indicated concentrations for an additional 48 h. Viability was assessed using the MTT (1-(4,5-dimethylthiazol-2-yl)-3,5-diphenyltetrazolium) test and absorbance was read at 562 nm using a microplate reader (BioKinetics Reader, EL340). Appropriate controls with DMEM only and MTT were used to determine background absorbance. Experiments were run in quadruplicate and repeated 3 times. Control cells were exposed to 1% DMSO which was not cytotoxic. The results are presented as the inhibitory concentration for 50% of cells (IC₅₀).

Cell cycle analysis

Lewis lung carcinoma and EAhy 926 endothelial cells were seeded in 6-well plates at 10⁵ cells/well. Twenty-four hours later, fisetin was added to the wells at the indicated

concentrations and the cells were incubated for 48 h. For each condition, detached and adherent cells were harvested, fixed for at least 30 min in 70% ethanol, and incubated with propidium iodide (50 µg/ml), sodium citrate (1 mg/ml) and RNase A (50 µg/ml) for 30 min in the dark. Nuclei DNA content was determined by flow cytometry (Coulter Epics Beckman, Germany) with red emission (FL-2 channel, 570 nm). After debris exclusion using forward/side scatter gating, 10^4 nuclei were acquired and analyzed using the WinMDI software. Cells with sub-G₁ DNA content were considered to be apoptotic.

Cell migration assay (scratch wound assay)

EAhy 926 endothelial cells were grown to confluence and a wound was introduced by clearing an area of the monolayer using a 100 µl pipette tip. Digital photographs of wounded areas were recorded from each well at a magnification of 100x (time 0 h). Following a change of medium, basic fibroblast growth factor (bFGF, 10 ng/ml, BD Biosciences) and fisetin at the indicated concentrations were added to the medium with 2.5% of FBS. After 24 h incubation, digital photographs of the wound areas were recorded for each well. Migration was evaluated by manually drawing the distance of the wound area (d) at T_{0h} and T_{24h}. Distance values were obtained using the ImageJ software [35]. Results were expressed as a percentage of the controls using the following formula: $100 \times [1 - (dT_{0h} - dT_{24h} \text{ of treated cells}) / (dT_{0h} - dT_{24h} \text{ of control cells})]$. Experiments were performed in triplicate for each concentration and were repeated 3 times.

Formation of capillary-like structures

Fifty µl of gel matrix solution was applied to each well of a 96-well plate and incubated for 30 min at 37°C. EAhy 926 cells (1×10^4) were suspended in 100 µl of medium, plated onto the gel matrix and incubated at 37°C. Adherent cells received bFGF (10 ng/ml) and fisetin at the

indicated concentrations. After a 24 h exposure time, *in vitro* angiogenesis was assessed by counting the number of capillary-like structures in each well at 100x magnification with a light microscope (Zeiss). The *in vitro* anti-angiogenic effect was calculated using the following formula: $100 \times [1 - (\text{number of capillary-like tubes in treated cells}) / (\text{number of capillary-like tubes in control cells})]$. Experiments were performed in duplicate for each condition and repeated 3 times.

Animal experiments

All animal experiments were ethically conducted, according to institutional, French and European guidelines, and were approved by the institutional animal welfare committee.

a) Matrigel plug angiogenesis assay. Fifteen 6-week old C57BL/6J female mice (Janvier, Le Genest Saint Isle, France) were randomly divided into five groups. LLC cells were trypsinized and resuspended at 3×10^7 cells/ml in serum-free medium. Aliquots of cells (0.1 ml, 3×10^6 cells) were mixed with 0.2 ml of phenol red-free Matrigel and injected into the right flank of mice. For the fisetin-treated groups, the cells were injected with four increasing concentrations of fisetin: 12.5, 25, 50 and 100 $\mu\text{g/ml}$ (or 22, 44, 87, 175 and 350 μM). Controls included cells with equal volumes of solvent, whereas the Matrigel mixed with the medium alone was used as a negative control. The Matrigel plugs were removed 14 days after the implantation, weighed and measured for haemoglobin content using the Drabkin's reagent kit according to the manufacturer's instructions (Sigma-Aldrich). Haemoglobin concentration was calculated based on a set of haemoglobin standards. The data are presented as mean \pm SEM from triplicate experiments.

b) Evaluation of antitumour activity in mice. In preliminary experiments with non tumoured C57BL/6J female mice, fisetin at 223 mg/kg intraperitoneally (i.p.) was found non toxic (based on body weights) when administered for 5 consecutive days in week 1 and for 5 consecutive days in week 2. Fisetin was dissolved in polyethylene glycol 200 (PEG₂₀₀)/DMSO (7:3; v:v) and injected i.p. in a volume of 0.1 ml. For antitumour evaluation, Lewis lung tumour fragments (about 3 mm³) were injected subcutaneously (s.c.) bilaterally into mouse flanks. Tumour growth was assessed every 2 days using bi-dimensional measurements with a caliper. Tumour volume (mm³) was calculated according to the formula: width² × length × 0.5 (mm). In the first experiment, fisetin was injected i.p. into 5 tumoured mice at 223 mg/kg on days 5 to 9 and days 12 to 16 post tumour implantation. In a second experiment, 20 mice were randomly divided into 4 groups. Fragments of LLC tumour (3 mm³) were injected bilaterally s.c. into the mouse flanks, and 4 days after tumour implantation, the mice were submitted to the following treatments: mice in the fisetin group were injected i.p. on days 4 to 8, 11, 12, and 14 with 223 mg/kg of fisetin dissolved in a 0.1 ml volume of PEG₂₀₀/DMSO (7:3; v:v); mice in the cyclophosphamide group were injected s.c. on days 4, 5, 7 and 8 with 30 mg/kg of cyclophosphamide dissolved in water; mice in the combination group were treated with both fisetin and cyclophosphamide as described above; mice in the control group were injected with both vehicles. The 30 mg/kg dose cyclophosphamide was based on previous work that showed that doses of 10-40 mg/kg can be administered daily for prolonged period without undue toxicity [36]. For comparison purposes, the maximum tolerated dose of cyclophosphamide in mice is between 186 to 220 mg/kg when administered as a single dose [37,38] and 170 mg/kg when given every 6 days [39]. Therefore, the 30 mg/kg dose for 4 days used in our experiments can be considered a low cyclophosphamide dose that was not toxic, based on body weight data. Tumour

measurements were recorded three times weekly; the mice were euthanized 15 days after tumour inoculation.

Microvessel density evaluation

Tumour tissues were harvested, weighed, frozen in isopentane, immersed in liquid nitrogen, and stored at -70°C until preparation of the histology slides. Ten-micron frozen tissue sections were placed on Superfrost Plus slides. Immunostaining of PECAM-1 (monoclonal rat antibodies anti-PECAM-1 (platelet endothelial cell adhesion molecule 1), clone MEC13.3; BDPharmingen, Le Pont-De-Claix, France) was performed using a three-step procedure as previously described [40]. In brief, the sections were washed three times in 1X phosphate buffered saline (PBS) and incubated for 10 min in 0.3% hydrogen peroxide/PBS. The slides were washed three times with 1X PBS, and incubated with 1% bovine serum albumin (BSA) at room temperature for 30 min. The sections were incubated with the rat primary antibodies anti-PECAM-1 (1:50) in a humidified chamber at 37°C for 1 h. After three washes in PBS, the slides were incubated for 30 min with biotinylated-secondary antibody with goat anti-rat IgG (1:400). After 3 rinses, slides were again incubated with the streptavidin-conjugated peroxidase according to the manufacturer's instructions (dilution 1/400). The 3,3'-diaminobenzidine (DAB) substrate was then added for 5 to 7 min until a brown precipitate was visible. Sections were rinsed several times in 1X PBS. Sections incubated with BSA instead of the primary antibodies were used as negative controls. Slides were counterstained with Gill's haematoxylin and treated with a 25% ammonia solution to generate a blue nuclear stain, dehydrated in graded ethanol solutions and xylene, and mounted with Eukit®. Microvessel density was evaluated under the microscope by counting 3 fields at a magnification of 100x on two different slides. Microvessel density was expressed as the number of PECAM-1 positive microvessels per mm^2 .

Statistical analyses

Results are expressed as the mean \pm SEM of at least 3 independent experiments. Comparisons between means were assessed using the Student t test for unpaired data. If unequal variance was observed, Welch's correction was applied. Comparisons between several groups were assessed using a one-way analysis of variance (ANOVA) followed by the Dunnett's multiple comparison test, using an appropriate control group as the reference. The statistical analyses were performed using the GraphPad Prism software. A P value < 0.05 was considered significant.

Results

Fisetin effects on cell viability, cell cycle and apoptosis of Lewis lung carcinoma (LLC) cells, endothelial cells (EAhy 926), and normal cells (NIH 3T3)

Fisetin induced a dose-dependent decreased viability in both Lewis lung carcinoma (LLC) and endothelial cells (EAhy 926), with IC_{50} s of 59 and 77 μ M, respectively, for a 24-h exposure time (Table 1). Interestingly, normal NIH 3T3 cells were found 3 times less sensitive to fisetin than either LLC or endothelial cells, with an IC_{50} of 210 μ M (Table 1). When the incubation time was increased to 48 h, the differential sensitivity between normal NIH 3T3 cells and LLC or EAhy 926 cells reached a 5-fold difference.

To characterize the mechanism of the cytotoxic/antiproliferative effect of fisetin, apoptosis was analyzed on LLC and endothelial cells. Table 2 shows the DNA cell cycle and sub G_1 distribution of fisetin-treated cells after 48 h exposure at the indicated concentrations. Fisetin induced apoptosis, measured as cells with sub- G_1 DNA content, in a dose-dependent manner in LLC cells. At low concentrations (22 and 44 μ M), fisetin induced apoptosis in 5% of LLC compared to 1% in control cells. Higher fisetin concentrations (175-350 μ M) induced higher levels of apoptosis (29%). Fisetin also induced a dose-dependent decrease in cells in G_1 . All tested concentrations of fisetin induced an accumulation of cells in the G_2/M phase (25-36%) compared to controls.

The fisetin effects on the cell cycle distribution of EAhy 926 endothelial cells differed markedly from the LLC cell line. Indeed, at low concentrations (22 and 44 μ M), fisetin already induced a higher percentage of apoptotic cells (11-15%), and at high fisetin

concentrations (175 and 350 μM) high levels of apoptosis were achieved (38% and 60%, respectively). As sub- G_1 apoptosis increased, the percent of cells in G_1 , S and in G_2/M phases decreased (Table 2). Contrary to LLC cells, no accumulation of fisetin-treated endothelial cells was observed in the G_2/M phase.

Fisetin inhibits angiogenesis *in vitro*

a) Effect of fisetin on migration of EAhy 926 endothelial cells. We next examined the possible antiangiogenic effects of fisetin on endothelial cell migration by using the scrape wound assay. Figure 2 illustrates that at 24 h post-wounding of confluent EAhy 926 endothelial cells, control cells migrated and totally filled the scraped area. Fisetin exposure at 22 and 44 μM (24 h), however, resulted in a significant dose-dependent decrease in EAhy 926 endothelial cell migration. The calculated IC_{50} for the anti-migration effect was $45 \pm 0.3 \mu\text{M}$ (mean \pm SEM from 3 independent experiments).

b) Effect of fisetin on capillary-like structure formation on Matrigel. The endothelial cell tube formation assay was used to investigate fisetin anti-angiogenesis effect *in vitro*. EAhy 926 endothelial cells plated on Matrigel with bFGF formed a capillary-like network within 24 h, as expected (Figure 3). Fisetin at 22, 44 and 87 μM prevented the formation of the capillary-like network in a dose-dependent fashion. The calculated IC_{50} for the inhibition of capillary-like structure formation was $52 \pm 7 \mu\text{M}$ (mean \pm SEM from 3 independent experiments).

Fisetin inhibits angiogenesis *in vivo*

We then investigated fisetin tumour angiogenesis *in vivo*. LLC cells were mixed with Matrigel with increasing concentrations of fisetin (44 to 350 μM) and injected s.c. into the right flank of mice. Fourteen days later, the mice were sacrificed and the Matrigel plugs removed, weighed, and evaluated for haemoglobin content. The Matrigel plugs were significantly smaller in the fisetin-treated groups compared to the controls. Matrigel plug weights decreased significantly as fisetin concentrations increased (Figure 4-A). To quantify angiogenesis, the haemoglobin content of the Matrigel plugs was assayed. As shown in Figure 4-B, fisetin treatment led to a dose-dependent decrease in Matrigel plug haemoglobin levels, which became significant at 350 μM . These *in vivo* results indicate that fisetin can decrease tumour angiogenesis.

Fisetin antitumour activity *in vivo*

To determine whether fisetin could inhibit tumour growth *in vivo*, fisetin was administered to two groups of 5 LLC tumour-bearing mice. Mice in the treated group were injected i.p. with fisetin at 223 mg/kg for 5 consecutive days during week 1, and for another 5 consecutive days in week 2. Mice in the control group received solvent on the same days as the fisetin-treated group. Preliminary experiments showed that fisetin alone was not toxic at this dose level and schedule of administration. On day 15, tumours from the mice treated with fisetin were 50% smaller than control tumours and appeared less vascularized than the controls (data not shown).

***In vivo* antitumour activity of the combination of fisetin and cyclophosphamide**

To optimize the *in vivo* anticancer effect of fisetin found above, fisetin was next combined with low dose cyclophosphamide, a cytotoxic drug reported to possess antiangiogenic properties [39]. Fisetin was administered i.p. at 223 mg/kg daily for 5 days in week 1 (days 4 to 8 post tumour implantation), followed by three injections on days 11, 12 and 14 (Figure 5, triangles). Fisetin treatment led to a 67% tumour growth inhibition compared to the controls (squares). Low dose cyclophosphamide was administered s.c. at 30 mg/kg on four days in week 1 only (days 4, 5, 7, 8), and led to a tumour growth inhibition of 66%, similar to fisetin treatment (Figure 5, diamonds). When fisetin and cyclophosphamide were combined at the same dose levels and schedules as used above, tumour volumes declined dramatically, showing 92% inhibition compared to controls on day 15 (Figure 5, solid circles). Over the two week treatment, this drug combination was not toxic, showing only a 4.6% loss in body weight, similar to that of the fisetin treatment alone (4.3%).

***In vivo* fisetin antiangiogenic effect**

To verify if the *in vivo* tumour growth inhibition was due to an antiangiogenic effect, tumour sections were stained using PECAM-1 antibodies. PECAM-1 was mainly expressed in endothelial cell membranes of microvessels, as expected (Figure 6-A, Control). The number of microvessels expressing PECAM-1 in the fisetin- and in the cyclophosphamide-treated tumours was significantly diminished compared to those in the control (Figures 6-A and 6-B). However, the treatment with the fisetin and cyclophosphamide drug combination led to an impressive and significant decrease in microvessel density, as depicted in Figures 6-A and 6-B.

Discussion

Although several phytochemicals have been shown to possess pharmacological properties of potential interest in cancer prevention and/or therapy, their activity in the tumour angiogenic process is presently not well understood [10-14,20]. Because we recently identified the dietary flavonoid fisetin as an interesting lead that can stabilize the cytoskeleton of endothelial cells *in vitro* at non cytotoxic concentrations [15], we were therefore interested to evaluate the *in vivo* antiangiogenic activity of this compound.

The fisetin antiproliferative/cytotoxic activity determined in this study on LLC and endothelial cells confirmed its cytotoxic activity reported on other cancer cell lines, e.g., in prostate [24], liver [23], colon [25], and leukaemia cells [21]. In this study, normal NIH 3T3 cells were also found to be about 3-fold less sensitive to fisetin than LLC or endothelial cells. It is of interest that the fisetin relative selectivity towards cancer and endothelial cells, compared to normal cells, has also been observed in other studies [20], and this selectivity was also observed on prostate cancer cells that were shown to be more vulnerable to fisetin compared to normal prostate cells [24]. This relatively non frequent cancer cell selectivity could therefore confer a valuable advantage of this compound for *in vivo* treatment.

We also observed that fisetin could block LLC cells in the G₂/M phase at low concentrations, and could induce apoptosis in endothelial cells also at low concentrations. These observations would suggest that fisetin could first act *in vivo* on endothelial cells forming the tumour vasculature and then cause apoptosis of cancer cells in the vicinity of the blood vessel. Fisetin-induced G₂/M cell accumulation has been previously reported along with decreased

activity of several cyclin-dependent kinases [21,23-25]. The signal transduction pathways involved in apoptosis include caspase 3 and increased p53 protein [23].

Our data clearly show that fisetin possesses *in vitro* antiangiogenic effects, preventing both the migration of endothelial cells and the formation of capillary-like structures at low micromolar concentrations. Previous work on *in vitro* antiangiogenic effects of fisetin has reported this effect at similar concentrations [20].

With regard to the relevance of the *in vitro* fisetin concentrations used in our experiments, the fisetin plasma concentrations achieved in mice are in the range of 10 μM after intraperitoneal administration of a dose of 223 mg/kg (Touil YS and Chabot GG, unpublished data). In rats, free fisetin plasma concentrations of 50 μM can also be achieved after an i.v. dose of 10 mg/kg [41]. These plasma concentrations are therefore in the range of the concentration used *in vitro* to show the antiangiogenic effects with the aglycone (free fisetin). In addition, it should be mentioned that because of the presence of 4 OH substituents on the fisetin molecule, glucuronide and sulphate conjugates are also present at high concentrations in plasma [41] (Touil YS and Chabot GG, unpublished data), and these metabolites could also play a role in the overall antiangiogenic effects observed *in vivo* in mice. It is of interest that flavonoid sulphates and/or glucuronides of closely related flavonoids (e.g., morin and quercetin) have recently been shown to display superior bioactivities compared to their aglycones (free forms) [42]. It should also be mentioned that mouse tumours usually have a high beta-glucuronidase and sulfatase activities that could hydrolyze locally the conjugates to release the aglycone within the tumour, and therefore contribute to the local antitumour effect [43].

We next investigated if these *in vitro* antiangiogenic effects could be translated *in vivo* using Lewis lung carcinoma bearing mice. Fisetin was found to cause significant tumour growth inhibition when used as a single agent at non toxic doses. The mechanism of action involved in the *in vivo* fisetin antitumour activity most likely involves an antiangiogenic effect, as evidenced by a significant reduction in microvessel density. Although fisetin's antiangiogenic activity has been previously reported *in vivo* in rabbit eyes, it should be mentioned that it was by direct application of an emulsion containing fisetin on the cornea [44] and not by systemic administration, as in the present study. To our knowledge this report is the first describing the fisetin's *in vivo* antiangiogenic activities after systemic administration in mice.

In an attempt to improve fisetin's *in vivo* antitumour effects, we next combined this flavonoid with low dose cyclophosphamide, because this cytotoxic agent has already been shown to improve antiangiogenic therapy [39,45]. This drug combination clearly led to an impressive improvement in antitumour effect with a 92% tumour inhibition at non toxic dosages of both agents. Although the fisetin-CPA drug combination is leading to a greater effect than either drug used alone, the magnitude of this effect could not be analyzed using the Chou and Talalay's method because measurements made with single doses of either drug in a combination can never alone determine synergism since the sigmoidicity of dose-effect curves and the exclusivity of drug effects cannot be determined from such measurements [46]. Because our data present only one dose level of either drug, we therefore cannot claim synergism, although there was a marked improvement in the anticancer activity of either drug at the single dose level used, as evidenced by tumour growth curves. Moreover, the histological examination of the treated tumours clearly showed that the microvessel density was significantly reduced in the tumours of mice that received the drug combination, thus

showing that an antiangiogenic effect was indeed involved in this impressive antitumour activity.

The precise molecular mechanism of action of the increased antitumour and antiangiogenic activity observed with the fisetin-cyclophosphamide drug combination is not precisely known for the moment. Although the antiangiogenic action of each compound alone is probably playing a major role in the improved activity of the combination, other factors could also be involved. For instance, a pharmacokinetic interaction would be possible, as was shown for the drug combination involving thalidomide and cyclophosphamide [37]. However, it should be mentioned that such a pharmacokinetic interaction was observed at a high dose of cyclophosphamide (220 mg/kg) [37], which is a 7-fold higher dose than the one used in the present study (30 mg/kg). Further studies will have to address this issue.

In addition to the stabilization of endothelial cells cytoskeleton [15], the antiangiogenic effect of fisetin and consequent antitumour activity could also involve the inhibition of urokinase plasminogen activator (uPA) in endothelial cells, as was recently reported [30]. UPA is over expressed in tumour vessels and is involved in extracellular matrix degradation responsible for endothelial cell migration and formation of new tumour blood vessels [47]. In addition to the Lewis lung carcinoma model, it is of interest to note that fisetin has recently been reported to be active in prostate cancer xenografts in nude mice through the inhibition of androgen receptor signalling, although angiogenesis was not investigated in this study [32]. Other potential mechanisms of action could involve the direct or indirect inhibition of other factors involved in the complex tumour angiogenic process.

Conclusions

The data reported here provide the first evidence that the dietary flavonoid fisetin can display antiangiogenic and anticancer activities *in vivo* in mice bearing Lewis lung carcinoma. In addition, the remarkable improvement in the anticancer and antiangiogenic activities of the combination of fisetin with low dose cyclophosphamide deserves further studies given the fact that cyclophosphamide is used in several anticancer drug regimens. Optimization of this drug combination by improved scheduling and/or pharmaceutical formulations is therefore warranted. It is proposed that the relatively non toxic drug combination studied in this work, associating a natural compound and a cytotoxic agent, could be useful in the treatment of solid tumours.

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Table 1 – Inhibitory concentration for 50% (IC₅₀) of Lewis lung carcinoma cells (LLC), EAhy 926 endothelial cells and normal NIH 3T3 cells. Cells were exposed to fisetin at various concentrations for 24 or 48 h, and viability was evaluated by the MTT test. Mean ± SEM of 3 independent experiments each performed in quadruplicate. Statistical significance assessed by one-way analysis of variance followed by the Dunnett’s multiple comparison test, using the IC₅₀ of NIH 3T3 cells as the reference. * = P < 0.001

Incubation time (h)	Inhibitory concentration for 50% (IC ₅₀) of cells (μM)		
	LLC	EAhy 926	Normal NIH 3T3
24	59 ± 9*	77 ± 9*	210 ± 14
48	27 ± 0.3*	28 ± 0.3*	136 ± 13

Table 2 – Cell cycle analysis of Lewis lung carcinoma and EAhy 926 endothelial cells treated with fisetin. Cells were exposed to the indicated concentrations of fisetin for 48 h. Cells were harvested, fixed, incubated with propidium iodide and analyzed by flow cytometry. Mean \pm SEM of 3 independent experiments.

Cell line	Fisetin	Percent cell in the indicated phase			
	Conc (μ M)	SubG ₁	G1	S	G ₂ /M
Lewis Lung Carcinoma cells	0	1 \pm 1	70 \pm 1	14 \pm 0.4	15 \pm 1
	22	5 \pm 1	51 \pm 1	19 \pm 0.3	25 \pm 1
	44	5 \pm 0.1	39 \pm 1	22 \pm 1	34 \pm 1
	87	12 \pm 0.1	26 \pm 1	26 \pm 1	36 \pm 1
	175	29 \pm 1	13 \pm 1	31 \pm 0.3	27 \pm 2
	350	29 \pm 1	14 \pm 1	29 \pm 0.1	28 \pm 0.3
EAhy 926 endothelial cells	0	4 \pm 1	66 \pm 2	18 \pm 1	12 \pm 2
	22	11 \pm 2	67 \pm 0.6	11 \pm 1	11 \pm 2
	44	15 \pm 1	65 \pm 1	12 \pm 1	8 \pm 0.3
	87	35 \pm 2	52 \pm 0.4	9 \pm 1	4 \pm 1
	175	38 \pm 1	49 \pm 1	8 \pm 1	5 \pm 2
	350	60 \pm 0.3	31 \pm 1	7 \pm 1	2 \pm 1

Figure legends

Figure 1 – Chemical structure of fisetin (3,3',4',7-tetrahydroxyflavone).

Figure 2 – Effect of fisetin on cell migration (scratch wound assay). EAhy 926

endothelial cells were grown to confluence and an area was cleared using a 100 µl pipette tip as described in the Methods. Digital photographs were recorded at a magnification of 100 X immediately after the wounding (Time= 0) and at 24 hours (T= 24 h) after the addition of bFGF (10 ng/ml) for the control, or with bFGF plus the indicated concentration of fisetin. Scale bar, 40 µm.

Figure 3 – Effect of fisetin on capillary-like structure formation. EAhy 926 endothelial cells were grown on Matrigel with bFGF (10 ng/ml) in absence (control), or presence of the indicated fisetin concentrations for a 24-h incubation period. Digital photographs recorded at a magnification of 100 X. Scale bar, 20 µm.

Figure 4 - *In vivo* Matrigel plug angiogenesis assay. Matrigel plugs containing Lewis lung carcinoma cells were implanted s.c. in mice with solvent (control=CTL) or with the indicated concentrations of fisetin. Fourteen days later, the Matrigel plugs were removed and weighed (A), and the content in haemoglobin was assessed (B), as described in the Methods. * P value < 0.05 and ** P value < 0.01 compared to controls (Dunnett's t test). The bar indicated "Matrigel" indicates the control without Lewis lung carcinoma cells.

Figure 5 - *In vivo* antitumour activity of the combination of fisetin with cyclophosphamide. Twenty mice bearing bilateral Lewis lung tumours were randomly

assigned to four groups of 5 mice as follows: control, solvent alone (squares); fisetin, 223 mg/kg i.p. on days 4 to 8 and days 11, 12, 14 (triangles); cyclophosphamide, 30 mg/kg s.c., on days 4, 5, 7, 8 (diamonds); and, the combination of cyclophosphamide and fisetin (solid circles), both administered at the same dose and schedule when used alone. Tumour volumes were determined as described in Materials and Methods. Mean \pm SEM. The * indicates a significant difference ($P < 0.05$) with the Control group, and the # indicates a significant difference with the Fisetin or the Cyclophosphamide group (ANOVA and Dunnett's multiple comparison test).

Figure 6 – Evaluation of microvessel density in Lewis lung carcinoma tumours. A)

Immunohistochemical evaluation of microvessels in Lewis lung tumours using antibodies to PECAM-1 as described in the Methods section. The tumours were treated *in vivo* with fisetin or cyclophosphamide alone, and with the combination of cyclophosphamide and fisetin. Scale bar, 100 μ m. **B)** Microvessel density (number of vessels per mm^2) in tumours after *in vivo* treatment with the solvent alone (control), with fisetin or cyclophosphamide (CPA) alone, or with the combination of fisetin and cyclophosphamide (CPA). Mean \pm SEM. The asterisks indicate a P value < 0.05 compared to controls (Dunnett's multiple comparison test).