**In vitro** Antiproliferative and Antiangiogenic Effects of Flavin7®

J. MOJŽIŠ1, M. ŠARIŠSKÝ1, M. PILÁTOVÁ1, V. VOHAROVÁ1, L. VARINSKÁ1, G. MOJŽIŠOVÁ2, A. OSTRO3, P. URDZÍK3, R. DANKOVČIK3, L. MIROSSAY1

1Department of Pharmacology, 2Department of Experimental Medicine, 3Second Clinic of Gynecology and Obstetrics, Faculty of Medicine, P.J. Šafárik University, Košice, Slovak Republic

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**Summary**

Flavin7 (F7) is a nutritional supplement often taken by cancer patients in Central Europe during chemo- and radiation therapy. In this study, investigation of the antiproliferative and antiangiogenic activities of this supplement were performed. Flavin7 showed antiproliferative activity in Jurkat as well as in HeLa cells. It significantly reduced the growth of both cancer cell lines at the doses of 200 μg/ml to 20 μg/ml (p<0.001 and p<0.01, respectively). In F7-treated Jurkat cells we found a significant increase in the fraction of cells with sub-G₀/G₁ DNA content, which is considered to be a marker of apoptotic cell death. Apoptosis was also confirmed by annexin V staining and DNA fragmentation. Furthermore, F7 at the doses of 100 μg/ml to 4 μg/ml inhibited endothelial cell migration and capillary tube formation what indicates its potential antiangiogenic properties. Flavin7 also inhibited the activity of matrix metalloproteinases (MMPs), preferentially MMP-9, at the doses of 100 μg/ml to 4 μg/ml. Our data suggest that F7 possesses marked antiproliferative and antiangiogenic properties *in vitro*. Further research is needed to elucidate also its *in vivo* activities.

**Key words**

Antiproliferative • Antiangiogenic • Polyphenols • Flavin7

**Corresponding author**

Jan Mojzis, Department of Pharmacology, Faculty of Medicine, P.J. Šafárik University, Trieda SNP 1, 04011 Košice, Slovak Republic. Fax: +421556428524. E-mail: jan.mojzis@upjs.sk

**Introduction**

Cancer is a major disease at worldwide level accounting for more than 7 million deaths/year. Unfortunately, current therapeutic modalities for advanced disease are of limited effectiveness. Alternative options for patients with malignancies include herbal treatments that have been used for many years throughout the world (Risberg *et al.* 1998). A wide variety of plant products is available in natural health products stores as dietary supplements for cancer patients.

Flavin7® (F7) is a nutritional supplement taken by cancer patients during radiation therapy or chemotherapy. It is popular mainly in Central Europe, but it is also marketed in Canada. It has been officially endorsed by the Hungarian Cancer Society as an effective product for enhancing the health of those subjected to chemotherapy (http://www.flavin7.com/hcs.php).

There are no *in vitro* or *in vivo* studies on the antiproliferative effects of F7 in cancer cells. However, flavonoids and resveratrol, the main active compounds in F7, display a remarkable spectrum of biochemical activities including those that might be able to influence processes that are dysregulated during cancer development. These include e.g. antioxidant activities (Cavallaro *et al.* 2003), the scavenging effect on activated carcinogens and mutagens (Ioannides and Yoxall 2003), the action on proteins that control cell cycle progression (Li *et al.* 2005) and gene expression (Gerritsen 1998). Moreover, it was found that some polyphenolic agents possess also antiangiogenic properties (Oak *et al.* 2005).

The purpose of this study was to assess *in vitro* antiproliferative and antiangiogenic properties of F7.
Methods

Reagents and drugs

MTT, 3-(dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was from Sigma-Aldrich Chemie (Steinheim, Germany), Cycle TEST™ PLUS DNA Reagent Kit, annexin V-FITC and propidium iodide were purchased from Becton Dickinson Biosciences (BDB, USA). Collagenase II was purchased from Invitrogen (USA). Flavin7® was a gift from Vita Crystal Slovakia Ltd (Slovak Republic).

Tumor cell lines

Jurkat cells (human acute T-lymphoblastic leukemia) and HeLa cells (human cervical cancer) were kindly provided by Dr. M. Hajduch (Olomouc, Czech Republic). The cells were routinely maintained in RPMI 1640 medium with Glutamax-I supplemented with 10 % fetal calf serum, penicillin (100 IU/ml) and streptomycin (100 μg/ml) (all from Invitrogen, USA), in the atmosphere of 5 % CO₂ in humidified air at 37 °C. Cell viability, estimated by trypan blue exclusion, was greater than 95 % before each experiment.

Cytotoxicity assay

The cytotoxic effect of F7 was studied by using colorimetric microculture assay with the MTT end-point (Mosmann 1983). Briefly, 8 x 10⁴ cells were plated per well in 96-well polystyrene microplates (Sarstedt, Germany) in the culture medium containing the tested chemicals at the doses of 200 μg/ml to 2 μg/ml. After 72 h of incubation, 10 μl of MTT (5 mg/ml) (Sigma, Germany) were added in each well. After additional 4 h, during which insoluble formazan was produced, 100 μl of 10 % sodium dodecylsulphate were added in each well and another 12 h were allowed for the formazan to be dissolved. The absorbance was measured at 540 nm using the automated MRX microplate reader (Dynatech Laboratories, UK). The absorbance of the control wells was taken as 100 % and the results were expressed as a percentage of the control.

Cell cycle analysis

The cell cycle distribution in cells treated with F7 was analyzed using Cycle TEST™ PLUS DNA Reagent Kit (BDB). Briefly, 5 x 10⁵ Jurkat cells were treated with F7 for 24 and 48 h. After treatment, cells were harvested, washed three times in citrate buffer and stained according to the manufacturer's instructions. Data acquisition was performed within one hour after staining on a FACS Vantage SE flow cytometer using CellQuest Pro software (both from BDB), information being stored for 5x10⁴ events per sample. The data were analyzed using Win MDI software. Percentages of cells corresponding to G₀/G₁, S and G₂/M cell cycle phases were calculated. The sub-G₀/G₁ fraction of cells was identified as the apoptotic population.

Annexin V/PI staining

Jurkat cells (5 x 10⁵) after Flavin7® exposure were washed twice in PBS and resuspended in 100 μl binding buffer (BDB). The cells were subsequently stained with annexin-V-FITC (AnV) and propidium iodide (PI) (both from BDB) according to the manufacturer's instructions. Immediately after staining, data acquisition was performed as described above. The data were analyzed using CellQuest Pro software (BDB).

DNA fragmentation assay

Treated and untreated cells (1 x 10⁶) were washed twice in PBS w/o calcium and magnesium. Cell lysis was performed in a buffer containing 10 mmol/l TRIS, 10 mmol/l EDTA and 0.5 % Triton X-100. Afterwards, proteinase K (1 mg/ml) was added and lysate was incubated for 60 min at 37 °C. Then, it was heated at 70 °C for 10 min followed by addition of RNAase (200 μg/ml) and another one hour incubation at 37 °C. Samples were subsequently transferred to 2 % agarose gel and run with 40 V at 3 h. DNA fragments were visualized by UV transluminator by ethidium bromide staining.

Endothelial cell isolation and cell cultures

Human umbilical vein endothelial cells (HUVECs) were isolated from freshly collected human umbilical cords by collagenase digestion of the umbilical vein interior according to the methods described by Marin et al. (2001). Cells were plated in 100x20 mm tissue culture dishes (Sarstedt, Germany) coated with 1.5 % gelatin. Cells were grown to confluence in cultivation medium. Primary cultures were harvested at confluence with 0.05 % trypsin – 0.02 % EDTA (Invitrogen, USA) and plated at a split ratio of 1:3 in tissue culture dishes. The cells were fed with fresh medium one day before each individual experiment. The endothelial identity of the cells was confirmed by their "cobblestone" morphology and CD31 expression. The cells were stained with a combination of CD45-FITC (BDB)/CD31-PE (Caltag, USA) monoclonal antibodies and analyzed by flow cytometry. Endothelial cells were
identified as CD31+CD45− cells and represented almost 100% of all cells in every primary culture.

**Endothelial cell migration assay**

The migratory activity of HUVECs was assessed using a “wound healing” assay. Subconfluent monolayers in 24-well plates were wounded with pipet tips giving acellular 1-mm-wide lane per well. After washing, cells were supplied with 1.5 ml cultivation medium in the absence (controls) or presence of F7. Cells were then allowed to migrate into the wound (empty space produced from the scratch) over a 24 h period. Cell migration was quantified as described by Cheung and Li (2002).

**In vitro angiogenesis assay**

The effect of the F7 on the capillary tube formation by endothelial cells was performed using the Fibrin Gel In Vitro Angiogenesis Assay Kit (Chemicon, USA) strictly following the manufacturer’s instructions. Briefly, HUVECs suspended in the Endomed medium (Biochrom, Germany) supplemented with VEGF and with or without F7 were seeded in fibrin-coated 96-well culture plates at concentration of 5 x 10⁵ HUVECs/100 μl/well. Tube formation was observed periodically over the time under a phase contrast microscope. Representative pictures shown in the Results section were taken at 24 h after F7 treatment.

**Gelatinase zymography**

Matrix metalloproteinases released into conditioned media were determined by gelatinase zymography according to the method of Newcomb et al. (2005) with minor modifications. Briefly, cells (5 x 10⁵) were seeded in 6-well plates in 2 ml cultivation medium for 24 h. Then, cells were washed with PBS and incubated in 1 ml of serum-free cultivation medium for additional 24 h in the absence (control) or presence of different dilutions of F7. Medium was collected, clarified of cellular debris by centrifugation for 20 min at 1000 g at −4 °C, and stored at −80 °C until analyzed. Proteins were electrophoresed under non-reducing conditions on 10 % polyacrylamide gels containing 1 mg/ml gelatin (Sigma-Aldrich). After electrophoresis, the gels were rewarmed in 2.5 % Triton X-100 (2 x 15 min), then incubated overnight at 37 °C in development buffer (50 mM Tris-HCl, pH 7.6; 10 mM CaCl₂; 50 mM NaCl; 0.05 % Brij35 [MP Biomedicals]). The gels were stained with 0.5 % Coomassie Brilliant Blue R-250 in 50 % methanol and 10 % acetic acid for 40 min at room temperature and then destained for 2 h in 50 % methanol and 10 % acetic acid. Proteolytic activity for MMP-2 in the gel was visualized as clear white bands at 68 kDa against a dark background and for MMP-9 at 92kDa. The gelatinase standard (Chemicon) was used as a positive control for gelatin zymography.

**Statistical Analysis**

For all experiments, mean values ± S.D. (from three experiments) were calculated using the Arcus Quickstat software package. To evaluate the statistical significance of observed differences between groups, Student’s t-test was employed. The statistical significance was considered to be present if p<0.05.

**Results**

**Cytotoxicity assay**

Survival of Jurkat as well as HeLa cells exposed to various F7 doses is shown in Table 1. F7 significantly decreased cell survival at the doses of 200, 100, 40 and

### Table 1. In vitro cytotoxicity data of F7 by the MTT assay (in %).

<table>
<thead>
<tr>
<th>Compound</th>
<th>200</th>
<th>100</th>
<th>40</th>
<th>20</th>
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<th>2</th>
</tr>
</thead>
<tbody>
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<td>F7 Jurkat cells</td>
<td>1***</td>
<td>1***</td>
<td>40***</td>
<td>75***</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>F7 HeLa cells</td>
<td>1***</td>
<td>1***</td>
<td>50***</td>
<td>76**</td>
<td>100</td>
<td>100</td>
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<tr>
<td>F7 HUVECs</td>
<td>5***</td>
<td>10***</td>
<td>80</td>
<td>100</td>
<td>100</td>
<td>100</td>
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***p<0.001, **p<0.01 vs. untreated cells (control)
20 μg/ml in both Jurkat and HeLa cell lines. Reduction in cell viability by ~50 % in comparison with the control was achieved at a dose of 40 μg/ml for both cancer cell lines. On the other hand, significant decrease in survival of HUVECS was observed only at the doses of 200 and 100 μg/ml (Table 1).

**Annexin V/PI staining**

In the control cells, less than 3.5 % of the early apoptotic cells (AnV⁻/PI⁻) were detected. Exposure (24 h) of cells to F7 at a dose of 40 μg/ml resulted in an evident increase in the proportion of early apoptotic cells (to 11.7 %). Furthermore, after 48 h of treatment we observed a significant increase in the number of early apoptotic cells as well as late apoptotic/necrotic cells (AnV⁻/PI⁻) and more than 73 % of the cells were stained with AnV or PI (Table 2).

**Cell cycle analysis**

Jurkat cells exposed to F7 at a dose of 40 μg/ml exhibited a time-dependent increase in the sub-G0/G1 fraction with the onset already after 24 h. After 48 h of incubation, more than 60 % of cells exposed to F7 were found to have sub-G0/G1 DNA content. These results indicate that F7 causes the appearance of the fraction of cells with sub-G0/G1 DNA content, which is suggestive of apoptosis (Table 3).

**DNA fragmentation assay**

DNA fragmentation reflecting the endonuclease activity characteristic of apoptosis was also analyzed. As shown in Figure 1, 72 h treatment with F7 at the doses of 200, 100, 40 and 20 μg/ml resulted in the formation of DNA fragments that could be visualized via electrophoretic examination as a characteristic ladder pattern.

**In vitro angiogenesis assay**

To evaluate potential antiangiogenic activity of
F7, a bioassay using HUVECs was applied. Ability of F7 to inhibit capillary tube formation by HUVECs grown on a fibrin gel matrix was examined. HUVECs in the Endomed medium supplemented with VEGF displayed a formation of capillary/tube-like networks after 24 h of incubation at 37 °C. F7 completely inhibited capillary tube formation by HUVECs in contrast to vehicle-treated control cultures. It should be noted that, although the signs of cytotoxicity were observed at the doses of 200 and 100 μg/ml, much lower concentrations (10 and 4 μg/ml) manifested no cytotoxicity, while a complete inhibition of tube formation was still observed (Fig. 2).

Endothelial cell migration assay

For the determination of potential antimigratory activities, F7 was tested in a wound closure assay. After wounding with a pipet tip, solvent controls reformed a confluent monolayer within 24 h of incubation. F7 was added at the doses from 100 to 2 μg/ml. In the presence of F7, a potent dose-dependent inhibition of endothelial cell migration was observed at the doses from 100 to 4 μg/ml (Fig. 3) (p<0.001; p<0.05).

Gelatinase zymography

To investigate the effect of F7 on MMP-2 and MMP-9 activity, gelatin zymography was performed. Gelatin zymography revealed that F7 reduced MMP-9 activity in HUVECs in a concentration-dependent manner. The active bands of MMP-9 gradually diminished when HUVECs were treated with different doses of F7 (from 4 to 100 μg/ml). Inhibitory effect of F7 on MMP-2 activity was observed only at the highest concentration (100 μg/ml) (Fig. 4).
Complementary and alternative medical therapies are commonly used in cancer patients (Nam et al. 1999). A large variety of herbal products is available in natural health products stores as dietary supplements for cancer patients. However, little is known about their efficacy, active principles and mode of action.

Flavin7® is derived from concentrated extracts of seven fruits (grape, blackberry, black cherry, black currant, elderberry, blackthorn and plum) chosen for their high concentration of polyphenols. One ml of F7 contains 22 mg of phenolic compounds such as myricetin, quercetin, kaempferol, isorhamnetine, (+)-catechin, (-)-epicatechin, malvidin-3-glucoside, caffeic acid, trans-reveratrol, cis-resveratrol, chrysin, galangin, apigenin, fisetin and luteolin. Although individual components of F7 have antioxidant, antiestrogenic, antitumor and many other properties, the in vitro evidence of anticancer activity for this polyphenol complex itself is still lacking.

In the last decade, advances in cancer research have enhanced our understanding of cancer biology and genetics. Among the most important of these is that the genes that control apoptosis have a major effect on malignancy through the disruption of the apoptotic process that leads to tumor initiation, progression, and metastasis. Therefore, one mechanism of tumor suppression by natural products may be to induce apoptosis. Our in vitro studies demonstrated that F7 significantly suppressed viability of cancer cells. To examine the mechanism that might account for the effects of F7 in cancer cells, we investigated its effects on cell cycle distribution and apoptosis. We observed F7-induced reduction in the proportion of cells in the S and G2/M phases with concomitant increase in the fraction of cells with sub-G0/G1 DNA content, which is considered to be a marker of apoptotic cell death. Apoptosis was also confirmed by annexin V/PI staining. In addition, DNA fragmentation was also clearly present as determined by agarose gel electrophoresis. The precise mechanism of F7 pro-apoptotic effect has not been elucidated yet. On the other hand, over the past few years, it has been shown that flavonoids can trigger apoptosis through the modulation of a number of key elements in cellular signal transduction pathways linked to apoptosis such as caspase pathway activation (Mak et al. 2006), inhibition of the major survival signals, Akt and extracellular regulated kinase (Granado-Serrano et al. 2006) or increase in intracellular free Ca2+ with calpain activation and mitochondria-mediated pathway (Das et al. 2006). Furthermore, the next component of F7 - resveratrol (3,5,4′-trihydroxystilbene), a naturally occurring phytoalexin, produced by a wide variety of plants such as grapes, peanuts, mulberries in response to stress, injury, UV irradiation, and fungal infection also possess pro-apoptotic properties (Ahmad et al. 2007; Li et al. 2006).

Angiogenesis is a highly regulated process that involves a complex cascade of events and plays an important role in tumor growth and metastasis. It is a key process in the promotion of cancer (Folkman 2003). Without angiogenesis, cancer cells cannot grow, and they remain dormant. Therefore, angiogenesis is a good target for cancer chemoprevention. It was documented that many natural health products inhibit angiogenesis. Among the known “natural” angiogenesis inhibitors, polyphenols seem to play an important role (Fotsis et al. 1997, Kanadaswami et al. 2005). However, the mechanism behind the antiangiogenic effect of polyphenols is not well understood.

This study tested the effect of F7 on cell biologic functions associated with angiogenesis. The functions examined were cell motility, cell proliferation and tube

**Fig. 4.** Inhibition of MMP-2 and MMP-9 activity. Conditioned medium of HUVEC treated with F7 (at the doses ranging from 100 to 4 μg/ml) was harvested after 24 h and subjected to non-reducing SDS-PAGE through a 10 % acrylamide resolving gel containing 1 mg/ml gelatin as described in Methods. F7 decreased gelatinolytic activity of enzyme MMP-9 as it is indicated by clear bands in the gel at lanes 2-6: F7 (at the doses of 100, 40, 20, 10 and 4 μg/ml). Line 1 is control and lane 6 is MMP-9 and MMP-2 standard. This is a representative gel picture of one of three separate experiments with similar results.
Flavin7 significantly suppressed above mentioned basic steps in angiogenesis. These results suggest that F7 could prevent tumor formation by inhibiting angiogenesis through the suppression of endothelial cell motility, proliferation and tube formation inhibition. As documented by Tan et al. (2006) similar results were obtained with quercetin, one of the main flavonoid in F7. They found that quercetin inhibited several important steps of angiogenesis including proliferation, migration, and tube formation of human endothelial cells in a dose-dependent manner.

Matrix metalloproteinases, particularly MMP-2 and MMP-9, play a crucial role in tumor invasion and angiogenesis. They are enzymes that break down extracellular matrix proteins to allow further differentiation and spread of endothelial cells during angiogenesis. Several reports indicate that MMPs has a major regulatory role in the initiation of angiogenesis (Folkman 2006). Recent evidence (Collen et al. 2003) has indicated the involvement of MMPs in the early stages of morphogenesis of endothelial cells on fibrin matrix. In this report, we demonstrated that F7 differentially regulated the activity of MMP-2 and MMP-9. Our results are consistent with other studies showing the ability of flavonoids as well as resveratrol to inhibit different MMPs (Vijayababu et al. 2006). These effects of the above mentioned polyphenols may partly explain our observation of inhibition of endothelial cells migration and capillary tube formation.

In summary, the present report describes for the first time potential anticancer effects of F7. We observed a marked in vitro effect of F7 on the growth, cell cycle and apoptosis of human tumor cell lines. Additional mechanisms such as the effects on growth, migration, tube formation by endothelial cells as well as MMPs inhibition were also observed. We conclude that F7 anti proliferative as well as antiangiogenic actions would effectively suppress the generation of cancer.

Conflict of Interest
There is no conflict of interest.

Acknowledgements
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