Effect of a Plant-derived Natural Compound, Flavin7, on the ERK Signaling Pathway in Immortalized Mouse Proximal Tubule Cells

EDIT NÁDASI[1,2], ISTVÁN EMBER[2] and ISTVÁN ARANY[1]

[1]*Department of Internal Medicine, University of Arkansas for Medical Sciences and
Central Arkansas Veteran HealthCare System, Little Rock, AR 72205, U.S.A.;*
[2]*Department of Public Health and Preventive Medicine, University of Pécs, 7624 Pécs, Hungary*

Abstract. *Background: Since MAP kinases represent an important pathway of transducing external stimuli to internal signals in cells, determining their possible role in cancer cells may offer a promising way for the treatment and prognosis of malignant diseases. Our previous experiments have shown that a flavonoid-rich solution, Flavin7, was able to diminish kidney tumor growth in vivo. Materials and Methods: Effects of Flavin7 on the MAPK signaling pathway were determined in immortalized mouse proximal tubule cells by determining cell viability, flow cytometric analysis, luciferase assays and Western blots. Results: At a nontoxic dose, Flavin7 markedly reduced phosphorylation of ERK and inhibited activity of its downstream targets such as Elk1 and CREB via inhibition of the ERK-kinase MEK1. Conclusion: Because of its ability to temporarily inhibit kidney tumor growth and activation of the MEK1/ERK pathway in vitro, further in vivo investigations may determine the potential role of Flavin7 in the treatment of malignancies.*

The mitogen-activated protein kinase (MAPK) cascade is a major signaling system by which cells transduce extracellular signals into intracellular responses. Many steps of this cascade are conserved, and homologs have been discovered in different species (1). The first three mammalian MAP kinases, ERK1, ERK2 and ERK3 were cloned in the early 1990s, facilitating the development of reagents for their study. It has become clear that ERK1 and ERK2 are among the protein kinases most commonly activated in signal transduction pathways. They have

particularly been linked to cell proliferation, but have important roles in many other events (2-4). In mammalian cells, ERK1 and ERK2, often referred to as p44 and p42 MAP kinases, are the archetypal members of the MAPK family. Therefore, determining the possible role of MAPKs in cancer cells may offer a promising way for treatment and prognosis of cancerous diseases.

According to recent results, activation of the ERK pathway is a frequent event in tumorigenesis. ERK has been implicated in cell initiation, tumor promotion and progression, invasion, metastasis, and regulation of apoptosis and angiogenesis, events that are essential for successful completion of developing a metastatic tumor (reviewed in 5). On the other hand, ERK activation is not unambiguously an advantage or a disadvantage for patients with cancerous diseases, since it has been shown to trigger cell proliferation and survival in normal cells, as well as in tumor cells.

Flavonoids, found in great quantity in fruit extracts, are secondary metabolites of superior plants exhibiting antitumor effects. They are known to exert antioxidant and antiproliferative effects on tumor cells (6). Recent studies have speculated that the classical antioxidant activity of flavonoids is unlikely to be the sole explanation for their cellular effects. This hypothesis is based on several lines of reasoning: i) flavonoids are extensively metabolized *in vivo*, thus, their redox potentials are significantly altered (7), and ii) the concentrations of flavonoids and their metabolites accumulated *in vivo* are lower than those of small of antioxidant nutrients (8). Investigations have indicated that flavonoids may selectively interact with the MAPK signaling pathway due to their ability to inhibit tyrosine kinase activity (9, 10).

A natural compound, Flavin7 (F7), composed of the extracts from seven different fruits, was investigated in our kidney tumor animal model (11). Ne/De tumor cells were transplanted underneath the renal capsule of 6- to 8-week-old Fisher344 rats and animals were treated with human

Correspondence to: Edit Nádasi, MD, Ph.D., Department of Public Health and Preventive Medicine, University of Pécs, 7624 Pécs, Szigeti u. 12, Hungary. Tel: +36 72 536394, Fax: +36 72 536395, e-mail: edit.nadasi@aok.pte.hu

dose-equivalent F7 solution according to the manufacturer's instructions. After two weeks of treatment rats were sacrificed and tumor growth was determined. F7 significantly ($p<0.05$) reduced tumor growth *in vivo*.

Accordingly, the aim of this study was to determine whether F7 influences the ERK signaling pathway in immortalized mouse renal proximal tubule cells.

Materials and Methods

Cell culture. The TKPTS cell line was obtained from Dr Bello-Reuss and grown in 5% CO_2 atmosphere at 37˚C as described by di Mari *et al.* (4). Parallel sets of logarithmically growing cells were treated with 50 μl, 100 μl, 200 μl, 300 μl and 500 μl F7 (total energy content 9.7 kJ, protein 0.07 g, fat 0.02 g, carbohydrates 0.16 g, total polyphenol 85 mg (flavonoid 75 mg, resveratrol 0.16 mg) 8 v/v% alcohol per 10 ml solution) (Crystal Institute Ltd, Eger, Hungary) per 2 ml cell culture medium.

Cell survival and FACS analysis. TKPTS cells were treated with various doses of Flavin 7 (50 μl, 100 μl, 200 μl, 300 μl and 500 μl F7/2 ml culturing medium). Viable cell count was determined by trypan blue (Sigma Chemical Co, St. Louis, MO, USA) exclusion in a hemocytometer after 24 hours of treatment. In addition, TKPTS cells treated with 100 μl F7 were collected for FACS analysis 24 hours after treatment. Briefly: TKPTS cells were collected after trypsinization and fixed in 70% ethanol overnight. After RNAse treatment, cells were incubated with 5 μg/ml PI and analyzed with a Becton Dickinson FACSCalibur analyzer (Becton Dickinson, Franklin Lakes, NJ, USA). The cell cycle profile was analyzed using the CellQuest software (Becton Dickinson, Franklin Lakes, NJ, USA).

Luciferase assay. pCRELuc plasmid containing four direct repeats of consensus CRE binding sites (Stratagene, La Jolla, CA, USA) was transiently transfected into TKPTS cells by using the GenePorter2 reagent (GenLantis, San Diego, CA, USA) together with a β-galactosidase plasmid (Promega, Madison, WI, USA) as described elsewhere (13). Similarly, the pFR-Luc reporter plasmid together with the pFA2-Elk1 trans-activator plasmid (Stratagene, La Jolla, CA, USA) was transiently transfected into TKPTS cells. Twenty-four hours after transfection cells were treated with Flavin 7 for various times (50 μl, 100 μl, 200 μl and 300 μl F7/2 ml culturing medium for 12 h). Luciferase activity was determined by using a Luciferase Assay Kit (Promega, Madison, WI, USA) while β-galactosidase was determined under control conditions and 18 hours after F7 treatment. The relative luciferase activity was measured and was normalized to the amount of activity detected for the co-transfected β-galactosidase plasmid (Invitrogen, La Jolla, CA, USA).

Western blots. Sets of logarithmically growing cells were treated with 100 μl F7 per 2 ml cell culture medium. Thirty minutes, 1 h, 2 h and 6 h after F7 treatment the medium was discarded, the cells were rinsed with 1xPBS and collected in a radioimmuno-precipitation assay (RIPA) buffer containing 50 μl/ml proteinase inhibitor cocktail (Sigma Chemical Co., St. Louis, MO, USA), 100 μmol/ml sodium orthovanadate (Sigma Chemical Co.) and 100 μg/ml phenylmethylsulfonyl fluoride (PMSF) (Sigma Chemical Co.) as described elsewhere (14). Protein content was measured using the
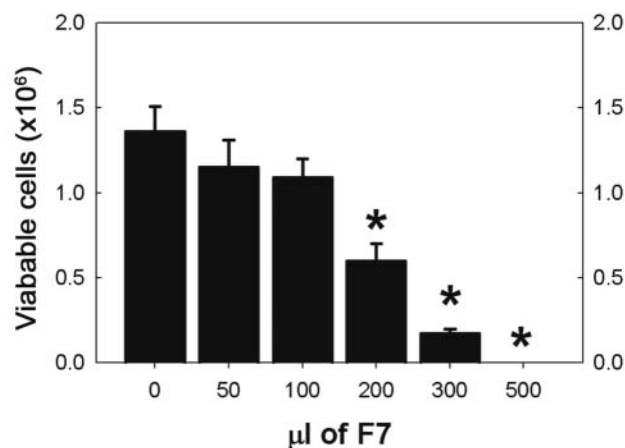

Figure 1. *Effect of F7 treatment on survival of TKPTS cells. Cell count was determined by trypan blue exclusion (mean±S.D., n=3, *p<0.001 compared to untreated control).*

Bio-Rad Protein Determination assay (Bio-Rad, Hercules, CA, USA) and 50 μg protein was blotted onto polyvinylidine fluoride membranes. The membranes were then hybridized with phospho-ERK1/2 (Thr202/Tyr204), ERK1/2 (Cell Signaling Technology, Beverly, MA, USA), phospho-MEK (Ser217/221) and MEK (Cell Signaling Technology) primary antibodies overnight in 5 ml 5% milk solution according to the manufacturer's instructions. After washing, the membranes were hybridized with a horseradish-peroxidase (HRP)-conjugated secondary antibody in 2 ml 5% milk solution for 45 min. Bands were visualized by using the enhanced chemiluminescence (ECL) method (Amersham, Piscataway, NJ, USA) and quantified by densitometry (UnScan-It 6.1; Silk Scientific, Orem, UT, USA).

Statistical analysis. Statistical differences between the treated and control samples were determined by Student's paired *t*-test. Differences between means were considered significant when $p<0.05$. Analyses were performed using the SigmaStat 3.5 software package.

Results

Effects of F7 treatment on cell viability. Treatment of the TKPTS cells with 50 μl, 100 μl, 200 μl, 300 μl and 500 μl F7/well showed that 200 μl, 300 μl and 500 μl F7 significantly reduced the number of cells in the culture while the 50 μl and 100 μl treatment had no such effects (Figure 1). FACS analysis of the TKPTS cells treated with 100 μl F7 showed that F7 did not change the cell cycle distribution of the cells significantly (Figure 2). Since no cytotoxic effect or change in viability could be seen when applying 50 μl or 100 μl F7, to assure the greatest therapeutic dose possible, we selected the 100 μl F7 dose for our further studies.

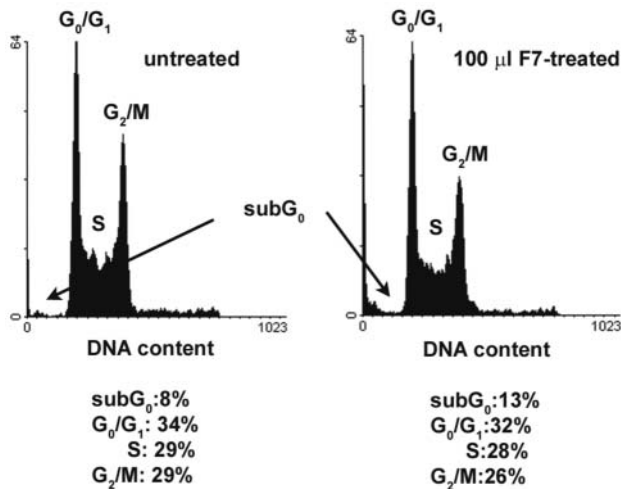*Effects of F7 treatment on ERK and MEK phosphorylation.* Next, we determined whether F7 treatment affected

Figure 2. *Effect of F7 on the cell cycle progression of TKPTS cells. TKPTS cells were treated with 100 μl F7 for 24 hours and FACS analysis was carried out as described in the Materials and Methods. The percentage of cells residing in the different stages of the cell cycle are given. Data are representatives of three independent experiments.*

phosphorylation of ERK. As shown in Figure 3, amounts of phospho-ERK were significantly and transiently reduced after F7 treatment in TKPTS cells (Figure 3 A and B). Since ERK is activated through its upstream kinase MEK (15), we were interested whether F7 affects ERK phosphorylation through its kinase. As the results of Western blotting show, F7 attenuates MEK phosphorylation (Figure 4 A and B) similar to ERK. On the other hand, total amounts of ERK and MEK were unchanged.

Effects of F7 treatment on downstream function of ERK. Activated ERK activates downstream targets that induce target-specific transcription, which is part of the survival signaling (13, 16). Elk1 is one of the downstream targets of ERK, activity of which could be followed by a trans-activating system, where the Elk activator plasmid initiates activity of a luciferase reporter plasmid. Accordingly, TKPTS cells were cotransfected with those plasmids and treated with 50 μl, 100 μl, 200 μl, 300 μl and 500 μl F7/2 ml culturing medium for 18 hours. As seen in Figure 5, Elk1 activity was significantly reduced after F7 treatment, similar to ERK phosphorylation (Figure 3). These observations suggest that ERK function is inhibited by F7 treatment.

The cAMP responsive element binding protein (CREB) is activated through phosphorylation by ERK/p90rsk (13, 17, 18). Activated CREB initiates a series of transcriptional events by binding to the promoters of CREB-responsive genes as part of the survival mechanism (19). To determine whether F7 also affects CREB-mediated transcription, TKPTS cells were transiently transfected with the CREB responsive pCRE-Luc
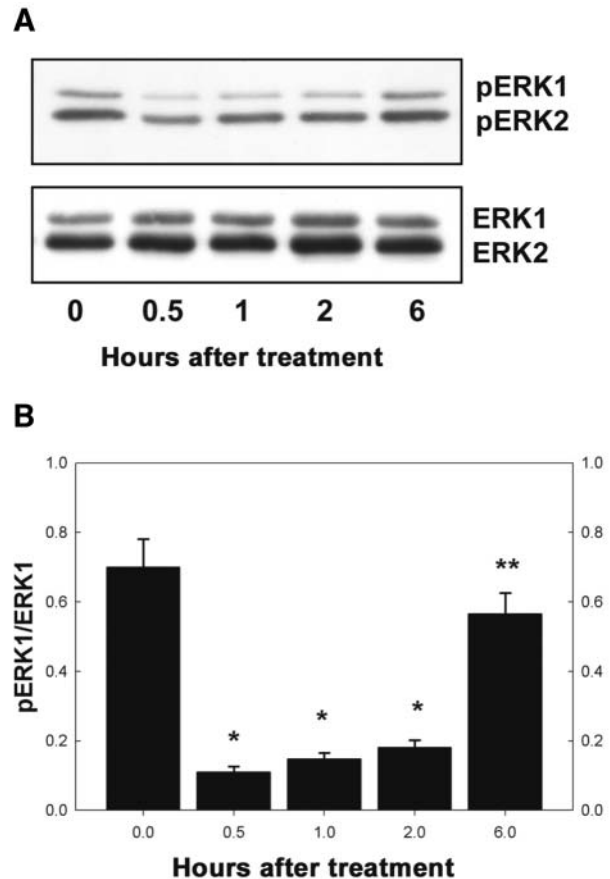

Figure 3. *Effect of F7 on ERK phosphorylation in TKPTS cells. (A) TKPTS cells were treated with 100 μl F7 for the times indicated. Phosphorylation of ERK was determined by Western blotting using an antibody that recognizes only the phosphorylated form. As loading controls, total ERK levels were also determined. Results shown are representative of three independent experiments. (B) Densitometry of the Western blots shown in (A). Ratios of pERK1/ERK1 are given (mean±S.D., n=3; *p<0.001 compared to the untreated control, **p<0.001 compared to the 0.5, 1.0 and 2.0 h treatments).*

plasmid. As is seen in Figure 5, F7 treatment significantly inhibited CREB-mediated transcription.

Discussion

Flavonoids comprise a large class of naturally occurring polyphenol plant compounds. The human diet usually contains approximately 1 g or more per day of flavonoids, a quantity providing pharmacologically significant concentrations in body fluids (20). Flavonoids were shown not only to inhibit tumor cell growth (11, 20) but also to induce cell differentiation (20). The inhibitory effects of flavonoids on the growth of malignant cells may partly be due to their suppressive effect on kinase activities involved in the regulation of cell proliferation (21, 22).
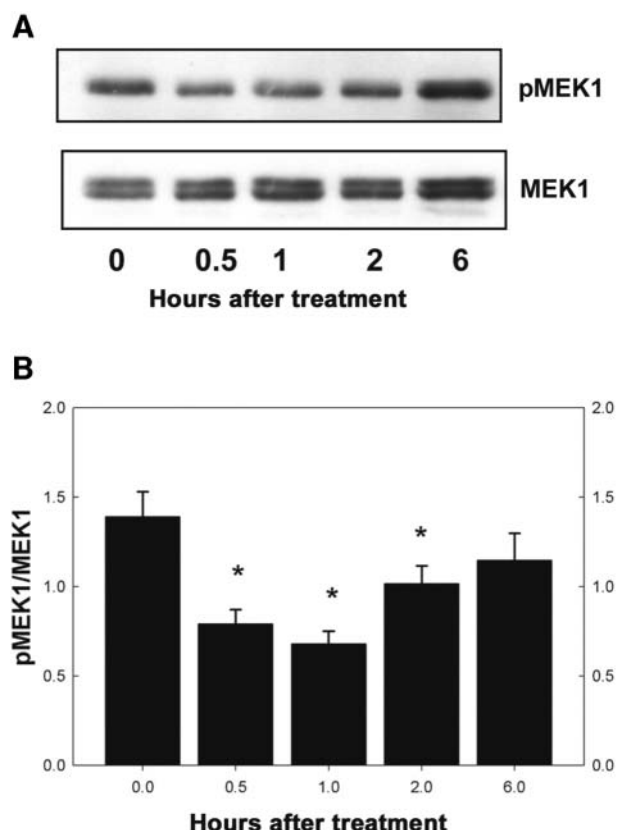
Figure 5. *Effect of F7 on downstream function of ERK. TKPTS cells were transiently transfected with either the pFR-Luc reporter plus pFA2-Elk1 trans-activator plasmids together with a β-galactosidase plasmid (open bars) or a pCRE-Luc plus β-galactosidase plasmid (filled bars) as described in Materials and Methods. After 24 hours, cells were treated with F7 for 12 hours and luciferase activity together with β-galactosidase activity was determined. Luciferase activity was calculated as ratio of luciferase activity normalized to β-galactosidase activity (mean±S.D., n=3; *p<0.001 compared to the untreated control).*

Figure 4. *Effect of F7 on MEK1 phosphorylation in TKPTS cells. (A) TKPTS cells were treated with 100 μl F7 for the times indicated. Phosphorylation of MEK1 was determined by Western blotting using an antibody that recognizes only the phosphorylated form. As loading controls, total MEK1 levels were also determined. Results shown are representative of three independent experiments. (B) Densitometry of the Western blots shown in (A). Ratios of pMEK1/MEK1 are given (mean±S.D., n=3; *p<0.001 compared to the untreated control).*

According to recent data, activation of the MAPK pathway such as ERK signaling is a frequent event in the development of cancerous diseases, though ERK phosphorylation is not unambiguously an advantage or a disadvantage for patient with cancerous diseases (5). The study of Ye *et al.* (23) has shown that the inhibition of the MEK/ERK pathway resulted in a significant enhancement of growth inhibition in MCF-7 breast cancer cells. So *et al.* (24) have demonstrated that citrus flavonoids effectively inhibited proliferation of the human breast cancer cell line MDA-MB-435 *in vitro*, especially when paired with quercetin, which is widely distributed in other foods.

Earlier, we reported that F7 treatment was able to reduce the growth of kidney tumor Ne/De transplanted into F344 rats. Furthermore, cancer-related weight loss was also reduced in tumor-bearing animals when treated with F7 (11). The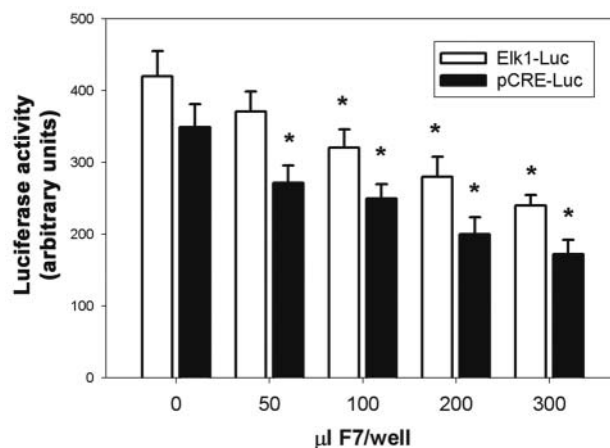 mechanism of this tumor-reducing effect was not investigated, but besides other mechanisms, effects on the MEK/ERK pathway may be suspected.

Our data suggest that F7, being a flavonoid-rich solution, possesses a protein kinase inhibitor activity that might be responsible for the observed inhibition of MEK and ERK phosphorylation in TKPTS cells (Figures 3 and 4). Whether F7 directly acts on MEK or on its upstream kinase(s) needs further investigation. Our data also showed that inhibition of ERK phosphorylation resulted in inhibition of its downstream function such as Elk1 or CREB activation (Figure 5).

ERK is involved in various cellular processes such as differentiation, proliferation and survival (5). Interestingly, inhibition of ERK and its function does not affect cell cycle progression as evidenced by FACS analysis (Figure 2). On the other hand, F7 also significantly inhibited CREB activity (Figure 5) probably through inhibition of ERK (13). Since CREB is an important element of survival signaling (13, 14, 18) these results suggest that F7 affects the survival pathway rather than the cell proliferation in renal tubular cells. The mechanism of these effects needs further study.

In conclusion, because of its ability to temporarily inhibit the activation of the MEK/ERK pathway *in vitro* and tumor growth *in vivo*, in case of malignancies which require MEK/ERK activation for survival (25), the natural compound F7 may have merit in the supportive therapy of cancer patients. Therefore, further *in vivo* and *in vitro* investigations are needed to determine the potential role of F7 in the treatment of malignant diseases.

Acknowledgements

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