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Short communication

APIGENIN INHIBITS GROWTH AND MOTILITY BUT INCREASES GAP JUNCTIONAL COUPLING INTENSITY IN RAT PROSTATE CARCINOMA (MAT-LyLu) CELL POPULATIONS [#]

MARTA CZERNIK, JOLANTA SROKA, ZBIGNIEW MADEJA and JAROSŁAW CZYŻ*
Department of Cell Biology, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, ul. Gronostajowa 7, 30-387 Kraków, Poland

Abstract: Apigenin (4',5,7,-trihydroxyflavone) is a flavonoid abundant in the common fruits, herbs and vegetables constituting the bulk of the human diet. This study was aimed at quantifying the effects of apigenin on the basic cellular traits determining cancer development, i.e. cell proliferation, gap junctional coupling, and motility, using the Dunning rat prostate MAT-LyLu cell model. We demonstrated that apigenin considerably inhibits MAT-LyLu cell proliferation and significantly enhances the intensity of connexin43-mediated gap junctional coupling. This effect correlates with an increased abundance of Cx43-positive plaques at the cell-to-cell borders seen in apigenin-treated variants. Moreover, we observed an inhibitory effect of apigenin on the motility of MAT-LyLu cells. The basic parameters characterising MAT-LyLu cell motility, especially the rate of cell displacement, considerably decreased upon apigenin administration. This *in vitro* data indicates that apigenin may affect cancer development in general, and prostate carcinogenesis in particular, via its influence on cellular activities decisive for both cancer promotion and progression, including cell proliferation, gap junctional coupling and cell motility and invasiveness.

Key words: Prostate cancer, Cell motility, Gap junctional coupling, Apigenin

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* Author for correspondence; e-mail: jaro@mol.uj.edu.pl

Abbreviation used: Cx43 – connexin43

INTRODUCTION

In a simplistic model of cancer development, deregulation of the signalling pathways determining cell proliferation and the dysfunction of gap junction proteins [1, 2] is often followed by the unrestricted (invasive) migration of cancer cells [3-5]. Neoplastic cells released from the controlling regime of the surrounding tissue can actively invade (intravasate) blood vessels and/or lymphatic vessels to reach the bloodstream and subsequently extravasate to form metastases by an expansive growth in the parenchyma of the target tissue [6]. Thus, aberrant proliferation, gap junctional coupling and cell motility seem to be cellular traits crucial for cancer development [7].

In recent years, plant polyphenolic compounds like phenolic acids and flavonoids drew considerable attention as chemopreventive dietary agents that are abundantly present in common fruits, herbs and vegetables. These substances have been shown to exhibit cancer-preventing activities in a variety of animal models of carcinogenesis, including prostate cancers [8, 9]. Among them, apigenin (4',5,7,-trihydroxyflavone) was shown to possess remarkable anti-inflammatory, anti-oxidant and anti-carcinogenic properties [10]. For example, an inhibitory effect of apigenin on UVB-induced skin carcionogenesis in hairless mice was demonstrated *in vivo* [11], and cell cycle arrest and/or apoptosis of normal and tumour cells was shown to be induced by apigenin in many *in vitro* studies [7, 10, 12-17]. This flavonoid was also shown to counteract the action of tumour promoters on gap junctional coupling [30]. Furthermore, it was found to have an inhibitory effect on tumour cell invasion [18-20]. With regard to prostate tumors, apigenin was revealed to induce growth arrest of some prostate carcinoma cells [16, 21]. However, a systemic quantification of the effect of apigenin on prostate cancer cell proliferation, intercellular communication and cell motility has not yet been performed.

A cellular model suitable for analyses of the effect of plant flavonoids on prostate carcinogenesis is the Dunning rat prostate carcinoma cell line MAT-LyLu. *In vitro*, MAT-LyLu cells are characterized by relatively high levels of Cx43-mediated gap junctional coupling, and display relatively high "contact-stimulated" motility, i.e. an induction of MAT-LyLu cell translocations is observed upon contact with neighboring cells [22]. Previously, a significant inhibition of cell motility in response to genistein, an isoconformer of apigenin, was observed [23]. Here, we systemically evaluated the effect of apigenin, administered at concentrations corresponding to those previously used to determine the effect of apigenin on basic cellular traits determining tumorigenesis [19, 24], on the cellular parameters relevant for prostate cancer development, i.e. cell proliferation, gap junctional intercellular coupling and motility.

MATERIALS AND METHODS

Cell culture

All the experiments were carried out on rat prostate cancer MAT-LyLu cells (the Dunning rat model) cultivated in Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma) supplemented with 5% foetal calf serum (Gibco) and antibiotics [22]. Stock cultures were harvested upon confluence with 0.25% trypsin, and seeded into culture dishes for endpoint experiments. Apigenin was administered to the cells from freshly made stock solution (4 mg/ml in DMSO). For the control experiments, DMSO (0.34% – an amount of solvent equivalent to 50 μM apigenin) was administered.

Proliferation and toxicity assay

Trypsinised MAT-LyLu cells were seeded into petri dishes (Nunclon, 35 mm) at an initial density of $7.5X10^3$ cells/cm^2. 24 hours after seeding, the culture medium was replaced with RPMI medium containing 25 or 50 μM apigenin, i.e. the concentrations corresponding to those used in previous studies on the biological activity of apigenin [19, 24], or a medium containing 0.34% DMSO. Cells were harvested 24, 48 and 72 hours after the administration of apigenin by trypsinisation, and the trypsin was subsequently inactivated with the original culture medium to preserve both the attached and suspended cells. Cell numbers were counted with a Bürker chamber. For the cytotoxicity tests, cells were cultured in control conditions or in the presence of 25 or 50 μM apigenin for 24 hours, followed by the addition of Hoechst 33342 (Sigma) and propidium iodide (PI, Sigma, final dye concentrations: 1 μg/ml for Hoechst and 50 μM for PI) to the original culture medium. The relative numbers of necrotic ($PI^+Hoechst^+$) and living ($PI^-Hoechst^+$) MAT-LyLu cells were estimated using a Leica DM IRE2 microscope. The results from three independent experiments (N = 3) were taken for the estimation of means and SEM values. The statistical significance was assessed with a paired t-student test against the DMSO control.

Estimation of gap junctional coupling and localisation of Cx43

Gap junctional coupling was measured as described previously [25]. In short, monolayers of acceptor cells were cultured in the presence of 0.34% DMSO (control) or 25 or 50 μM apigenin for 24 hours, followed by the addition of calcein- and DiI-labelled donor cells plated at a ratio of l:50. After a 3 hour-long incubation, calcein transfer from the donor to recipient cells was evaluated using a Leica DM IRE2 microscope. Homologous gap junctional coupling was quantified as the number of recipient cells that obtained calecin from one donor cell (coupling ratio – c_r). A dye transfer from at least 50 donor cells per coverslip was analysed in three independent experiments (N = 3) performed for each experimental condition. For the immunofluorescence studies, confluent cells on coverslips were fixed, stained with primary (mouse anti-Cx43 IgM, Sigma) and secondary antibodies (Cy3-conjugated goat anti-mouse IgM, Dianova) as described previously [26], and counterstained with bis-benzimide (Hoechst,

0.5 μg/ml). The abundance of Cx43-positive plaques in at least three independent specimens (N > 3) was semi-quantitatively assessed for each culture variant. The intensity of punctate Cx43-specific fluorescence was rated from (+) for immunoreactivity characteristic for the controls, to (+++) for a high abundance of Cx43-specific plaques.

Immunoblotting analysis

Monolayers of MAT-LyLu cells cultured in the presence of 0.34% DMSO (control) or 25 and 50 μM apigenin for 24 hours were washed, harvested using a cell scraper, centrifuged and dissolved in lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.02% sodium azide and a proteinase inhibitor cocktail (20 μl/ml). The whole cell extract was then centrifuged and the protein content of the lysates was determined with the Bradford method. Cellular proteins (40 μg/lane) were applied to 12.5% SDS-polyacrylamide gels, followed by transfer to nitrocellulose. Blots were exposed to the primary antibody (rabbit anti-connexin43) and mouse monoclonal anti-actin antibody (both from Sigma) followed by detection of the antibodies using HRP-labeled secondary antibodies (Bio-Rad) and an ECL Western detection system (Pierce, Rockford, IL). 3 independent Western blot assays were performed.

Time-lapse monitoring of cell movements

MAT-LyLu cells were plated into culture flasks (Corning, 25 cm^2) at initial cell densities chosen to compensate for the inhibitory action of apigenin on cell proliferation (200 to 400 cells/mm^2). The movement of individual cells in the presence of DMSO or apigenin (25 and 50 μM) in RPMI medium with supplements was performed 24 hours after the administration of agents using a computer-assisted data acquisition system (recording time: 4 hours, with 5-minute time intervals at 37ºC). The tracks of individual cells were determined from the series of changes in the cell centroid positions, and cell trajectories (> 50 cells, three independent experiments) were pooled and statistically analyzed [22, 27].

RESULTS AND DISCUSSION

Apigenin inhibits the proliferation of MAT-LyLu cells

Apigenin was previously demonstrated to attenuate prostate carcinogenesis via the inhibition of cancer cell proliferation [21]. Therefore, we first concentrated on the effect of apigenin administered at the physiologically relevant concentrations of 25 and 50 μM [13, 24, 28] on the proliferation and motility of MAT-LyLu cells. An inhibition of MAT-LyLu cell mitotic activity was observed in the presence of apigenin. This was illustrated by the shapes of the growth curves drawn for the populations cultured in control conditions and in the presence of apigenin. While an exponential growth of MAT-LyLu cells was observed under the control conditions, a complete growth arrest was induced by

25 μM apigenin. Furthermore, a slight decrease in cell numbers in the presence of 50 μM apigenin could be seen, indicating a marginal cytotoxic effect exerted by apigenin at this concentration (Fig. 1A). In fact, cytotoxicity tests performed to discriminate between growth inhibition and the toxic effects of apigenin revealed a slight, but significant ($p < 0.05$) increase in the numbers of PI-positive (necrotic) cells in cultures incubated in the presence of 50 μM apigenin (7.6 ± 1.4%) compared to the DMSO control (1.6 ± 0.4%). On the other hand, no toxic effect of apigenin administered at the lower (25 μM) concentration on MAT-LyLu cells could be observed, since the fraction of dead cells (2.2 ± 0.4%) in the 25-μM apigenin-treated populations corresponded to control value.

These observations are consistent with earlier data indicating that apigenin induces cell cycle arrest in normal and tumour cells. For example, the G_1 and/or G_2/M block of cell proliferation of normal and transformed cells has been described [12, 13, 29] to result from the activation of p53 and p21/WAF1 [12, 13], and their interference with the signalling pathways regulating the function of cyclins, such as those dependent on MAPK [21]. Thus, the interference of apigenin with MAT-LyLu cell growth indicates that the chemopreventive effect of this flavonoid on prostate carcinogenesis depends on its interference with cancer cell proliferation [10, 21].

Apigenin augments the intensity of gap junctional coupling in MAT-LyLu cell populations

Gap junctions are intercellular channels that enable the direct intercellular exchange of small metabolites. In the initial stages of tumour development, the inhibition of connexin expression and gap junctional coupling seems to promote tumour development in a coupling-dependent and/or -independent manner [7]. Since apigenin was previously shown to modulate the function of gap junctions [24, 28], we performed semi-quantitative analyses of the effect of apigenin on gap junctional coupling intensity in MAT-LyLu cell populations. A significant increase in the gap junction-mediated transfer of calcein was observed in cell populations cultured in the presence of 25 μM apigenin, compared to the control (Fig. 1B, see 1D cf. C). The c_r value, which represents the averaged number of recipient cells that obtained calcein from one donor cell, increased from 7.7, calculated for control variants, to 26.7 for populations cultivated in the presence of 25 μM apigenin (Fig. 1B).

The effect of apigenin on coupling intensity was correlated with an increase in the numbers of "mature" Cx43-specific plaques at cell-to-cell interfaces observed in MAT-LyLu cell populations cultured in the presence of 25 and 50 μM apigenin (Fig. 1G, H cf. F, Tab. 1). On the other hand, Western blot analyses did not reveal any increase in the Cx43 protein levels induced by apigenin (Fig. 1I).

Impaired intercellular communication via gap junctions is considered to facilitate the release of a potentially neoplasic cell from the controlling regime of the surrounding tissue, leading to tumour promotion [1]. Neoplastic cells usually display decreased levels of connexin expression and/or gap junctional coupling [7].

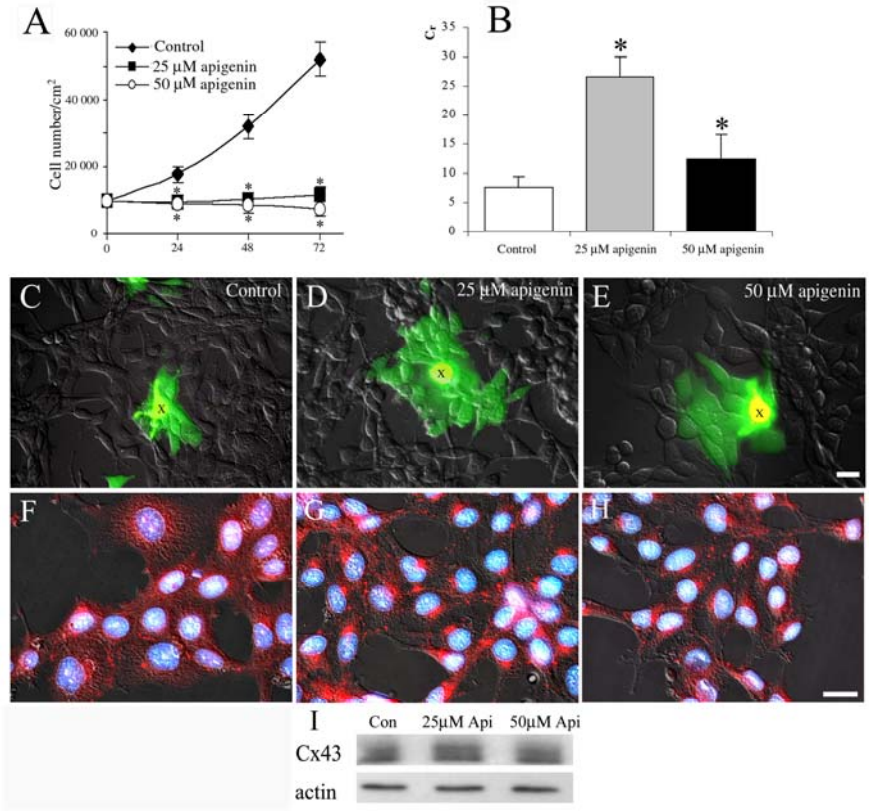

Fig. 1. The effect of apigenin on the proliferation of MAT-LyLu cells (A) and gap junctional coupling in MAT-LyLu cell populations (B). Apigenin inhibits the proliferation of MAT-LyLu cells in a dose-dependent manner as demonstrated by the growth curves (means ± SEM, A). This effect is correlated with an increased intensity of gap junctional coupling, illustrated by an increase in the c_r parameter value, representing the number of calcein-positive recipient cells per one donor cell analysed 3 hours after donor cell seeding (B). C-E depict the spreading of calcein (green) from calcein/DiI (yellow-marked with **x**) loaded cells to acceptor cells in MAT-LyLu populations cultured in control (0.34% DMSO) conditions (C), and in the presence of 25 (D) and 50 µM (E) apigenin. This correlated with a higher abundance of gap junctional plaques at cell-to-cell interfaces in apigenin-treated (G, H) compared to control variants (F; for semi-quantitative analysis see Tab. 1). However, no significant effect of apigenin on Cx43 protein levels was observed (I). Bars represent means ± SEM. *$p < 0.05$ as determined by the paired-t-Student test obtained from three independent experiments (A), and from at least 50 donor cells per coverslip performed in triplicate (B). Scale bar = 20 µm.

The data presented here, indicating that apigenin augments the intercellular transfer of metabolites in MAT-LyLu prostate carcinoma cell populations, suggests that this flavonoid may inhibit prostate carcinogenesis not only via proliferation arrest but also through an enhancement of intercellular

communication. A corresponding effect of apigenin on gap junctional coupling was described by Chaumontet *et al.* [24], who demonstrated that apigenin enhanced a dye transfer in populations of liver epithelial (REL) cells. Moreover, increased amounts of Cx43 were observed in apigenin-treated REL cells [24]. Our data indicates that apigenin enhances gap junctional coupling in MAT-LyLu populations without considerable effect on the Cx43 protein levels. Thus, the increased abundance of Cx43-positive plaques at cell-to-cell contacts in apigenin-treated cell populations indicates a shift in the proportion between the surface (active) and cytoplasmic pools of Cx43.

Tab. 1. A semi-quantitative analysis of the effect of apigenin on the abundance of Cx43-positive plaques in rat prostate MAT-LyLu cell populations (for details see the Materials and Methods section).

Concentration of apigenin [μM]	0	25	50
Intensity of punctate Cx43-specific fluorescence	(+)	(+++)	(++)

(+) intermediate abundance (control); (++) high abundance; (+++) very high abundance

On the other hand, the increase in the intensity of gap junctional coupling observed in MAT-LyLu cell populations incubated in the presence of 50 μM apigenin (Fig. 1E cf. C), although statistically significant when compared to the control, remains considerably lower than in variants treated with 25 μM apigenin (Fig. 1B). Since a marginal cytotoxic effect of 50 μM apigenin on MAT-LyLu cells was observed, it is conceivable that a partial closure of gap junctions may occur in sub-lethally affected cells at high apigenin concentrations to prevent a "by-stander" effect resulting from the intercellular spreading of erroneous signals via gap junctions. A modulation of the cytotoxicity of apigenin by a coupling status of cell populations was previously described for HeLa cells [28].

The motility of MAT-LyLu cells is reduced by treatment with apigenin

According to the common model of carcinogenesis, a deregulation of pathways regulating cancer cell proliferation and gap junctional coupling is followed by the acquisition of the capability of unrestricted, invasive migration by cancer cells [3]. The induction of cell migration is a key step in cancer development, and a correlation between the motility of tumour cells and their invasive potential has been demonstrated [31]. It was previously shown that apigenin inhibits motility and reduces the invasive potential of HeLa Cx43 cells [19]. Therefore, we further concentrated on the effect of apigenin on the motility of MAT-LyLu cells.

A morphological examination revealed that MAT-LyLu cells remained well attached and spread on the substrate in the presence of apigenin regardless of its concentration (Fig. 1), whereas time-lapse analyses revealed a high motile activity of MAT-LyLu cells under control conditions (Fig 2; Tab. 2). This is characteristic for these "contact-stimulated" cells when cultured at high densities [22].

Apigenin significantly inhibited MAT-LyLu motility in a dose-dependent manner as illustrated by circular diagrams depicting MAT-LyLu cell trajectories in control conditions (Fig. 2A) and in the presence of apigenin (Fig. 2B, C). Most importantly, the value of the length of cell displacement, calculated for cells cultured in the presence of 25 and 50 μM apigenin, respectively reached only 73 and 66% of the control (Fig. 2D; Tab. 2).

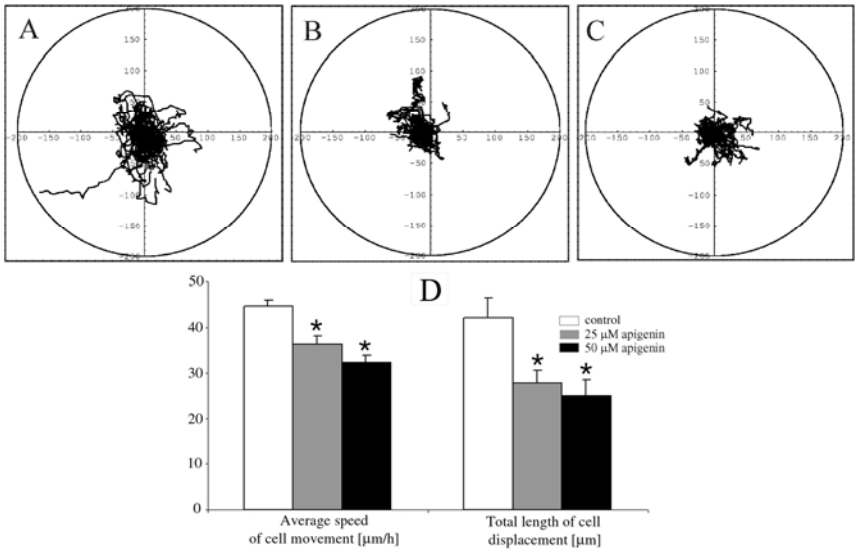

Fig. 2. The effect of apigenin on the motility of MAT-LyLu cells. The trajectories of cells cultured in the presence of 0.34% DMSO (A), and 25 (B) and 50 μM (C) apigenin are depicted in the form of circular diagrams (axis scale in μm) drawn with the initial point of each trajectory placed at the origin of the plot. The quantification of the basic parameters of cell motility is presented (D). Apigenin significantly inhibits the speed of MAT-LyLu cell movement and displacement in a dose-dependent manner. *$p < 0.001$ determined with the Mann-Whitney test of the data obtained from three independent experiments (N = 3) performed against the DMSO control.

While the cytostatic activity of apigenin was demonstrated in many normal and tumour cellular systems, only a few studies focused on its interference in cell motility, i.e. a factor determining tumour invasion [18, 19]. It was previously shown that human cervical carcinoma (HeLa Cx43) cells react to apigenin by inhibiting cell translocation, which correlates with impaired cell invasion [19]. Our results, which reveal that apigenin inhibits the motility of prostate carcinoma cells, suggest that this flavonoid may also interfere with prostate cancer cell invasion. Interestingly, a similar inhibition of MAT-LyLu cell motility was observed upon the administration of genistein [23].

In summary, this paper describes a novel experimental approach based on the *in vitro* quantification of the proliferation, migration and gap junctional coupling in

carcinoma cell populations as an efficient tool for the elucidation of the basic mechanisms of the chemopreventive action of flavonoids. We demonstrated that apigenin exerts a profound cytostatic effect on Dunning rat prostate carcinoma MAT-LyLu cells, which are an *in vitro* cellular model of prostate cancer [22, 32]. This effect is correlated with an augmentation of gap junction-mediated dye transfer in MAT-LyLu cell populations. As the disturbance of systems controlling cancer cell proliferation and the intensity of the transfer of small metabolites through gap junctions is crucial for the promotion of cancers including prostate carcinogenesis [7], the presented results indicate that apigenin may interfere with early prostate cancer cells via a combined action on cell proliferation and gap junctional coupling. On the other hand, the inhibition of MAT-LyLu cell motility observed upon apigenin treatment demonstrates that this flavonoid also seems to interfere with cellular invasive potential [32].

Tab. 2. A summary of the quantitative data showing the effect of apigenin on the motility of MAT-LyLu cells.

Parameters (means ± SEM)	Concentration of apigenin [μM]		
	0 (DMSO)	25	50
Total length of cell trajectory [μm]	178.3 ± 5.82	140.59 ± 7.85$^{\#}$	129.07 ± 5.19$^{\#}$
Average speed of cell movement [μm/h][1)]	44.6 ± 1.46	36.15 ± 1.96$^{\#}$	32.27 ± 1.3$^{\#}$
Total length of cell displacement [μm]	42.13 ± 4.37	27.69 ± 2.72$^{\#}$	24.9 ± 3.4$^{\#}$
Average rate of cell displacement [μm/h] [2)]	10.54 ± 1.1	6.92 ± 0.68$^{\#}$	6.23 ± 0.85$^{\#}$
Coefficient of movement efficiency [CME][3)]	0.23 ± 0.018	0.20 ± 0.017	0.17 ± 0.036

The tracks of individual cells were determined from the series of changes in the cell centroid positions, and cell trajectories (> 50 cells, three independent experiments) were pooled and analyzed. [1)]Average speed of cell locomotion is defined as total length of cell trajectory/time of recording (4 h). [2)]Average rate of cell displacement is defined as total length of cell displacement from the starting point to the final cell position/time of recording (4 h). [3)]Ratio of cell displacement to cell trajectory length. CME would equal 1 for a cell moving persistently along one straight line in one direction and 0 for a randomly moving cell. Statistically significant (Mann-Whitney test) probe vs. control without apigenin; $^{\#}p < 0.001$.

Furthermore, an experimental design based on the systemic quantification of the cellular traits involved in prostate carcinogenesis seems suitable for the elucidation of mutual interrelations between cellular traits decisive for carcinogenesis. In particular, we demonstrate that apigenin enhances gap junctional coupling in MAT-LyLu populations without considerable effect on the Cx43 protein levels. As gap junctional coupling depends on the stability of cell-to-cell contacts, the observed inhibition of intercellular translocations by apigenin (Fig. 2) may result in the stabilization of gap junctional plaques (Fig. 1F-H), which facilitates gap junctional intercellular coupling in prostate carcinoma cell populations (Fig. 1C-E). Research aimed at the elucidation of the effect of cell motility on the intensity of intercellular communication in MAT-LyLu

cell populations is now under way to verify this hypothesis. It is now a matter of debate whether this correlation might be of special importance as a tumour stage-specific function of gap junctions. Gap junctional coupling, while inhibiting cancer cell promotion, was also suggested to alleviate tumour invasion in some cellular models (for review see [7]). Therefore, further systemic evaluation of the effect of flavonoids on gap junctional coupling during prostate cancer promotion and progression is necessary for a better understanding of the mechanisms governing the postulated chemopreventive activity of these agents.

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