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Suppression of Taxanes Cytotoxicity by Citrus Flavonoid Hesperetin in PPC-1 Human Prostate Cancer Cells

KATRIN SAK[1], HELEN LUST[1], MARJU KASE[2,3], MARIKA SAAR[4] and JANA JAAL[1,2]

[1]*Clinic of Hematology and Oncology, Institute of Clinical Medicine, University of Tartu, Tartu, Estonia;*
[2]*Department of Radiotherapy and Oncological Therapy, Tartu University Hospital, Tartu, Estonia;*
[3]*East-Tallinn Central Hospital, Tartu, Estonia;*
[4]*Pharmacy, Tartu University Hospital, Tartu, Estonia*

Abstract. *Background/Aim: More than half of prostate cancer patients use, in addition to conventional therapies, some kind of complementary medicine, including flavonoid-rich products. However, knowledge about the co-effects of flavonoids with cytotoxic chemotherapies is still rather poor. Therefore, this study was undertaken to assess the cytotoxic activity of flavonoids and their interactions with taxanes in human advanced prostate cancer cells. Materials and Methods: Cytotoxicity of different flavonoids and their effects on the efficacy of docetaxel and cabazitaxel were studied in the human metastatic prostate cancer cell line PPC-1, using MTT colorimetric assay. Results: Both taxanes suppressed the viability of PPC-1 cells with IC_{50} values in the nanomolar range. Tested flavonoids exerted cytotoxic activity only at high micromolar concentrations or revealed no remarkable effect on cell survival. Simultaneous treatment of cells with taxanes and flavonoids baicalein, chrysin, luteolin, fisetin, quercetin, genistein or daidzein did not lead to any change in chemotherapy-induced cytotoxicity. However, simultaneous exposure of cells to hesperetin and taxanes resulted in 9.8- and 13.1-fold reduction in cytotoxicity of docetaxel and cabazitaxel, respectively. Conclusion: Flavonoid hesperetin remarkably suppressed the cytotoxic efficacy of taxanes in prostate cancer cells. Therefore, caution is required from prostate cancer patients who take hesperetin-containing oral supplements.*

Prostate cancer is the most common malignancy among men worldwide. According to GLOBOCAN, about 1.1 million new cases and 307,000 deaths were recorded in 2012, making it thus the third-leading cause of cancer-related death in men (1, 2). Although treatment with radiation and surgery can be curative in localized organ-confined prostate tumors, more than one third of patients progress to metastatic disease (3, 4). For these men, androgen deprivation therapy is the standard of care (3). However, after initial remission in most patients, tumor eventually develops into castration-resistant disease (5). Chemotherapy with taxane (docetaxel or cabazitaxel) is currently considered one of the effective treatment options for advanced castration-resistant prostate cancer that exerts its anti-tumor activity through direct antimitotic effects as well as by suppressing the microtubule-dependent trafficking of proteins like androgen receptors (6). Despite effective anti-cancer therapies, more than half of prostate cancer patients use additionally some kind of complementary medicines (CM), whereas the majority of patients believe that CM are "helpful" or "very helpful" for their condition (7). This is somewhat surprising since conclusive evidence regarding the use of CM is still lacking and current knowledge relies mostly on few experimental studies performed with cancer cell lines and/or animal models. Furthermore, interactions of CM with conventional anti-cancer therapies, including taxane-based chemotherapy in prostate cancer, are largely unknown.

Previous studies have reported that the most commonly used CM among cancer patients are vitamins and different herbal products (8, 9). Among those, interest has been lately focused to plant secondary metabolites, flavonoids, which can be found abundantly in fruits and vegetables, seeds and herbs. Based on their structural peculiarities, flavonoids can be divided into six subclasses: flavonols, flavones, flavanones, flavanols or catechins, isoflavones and anthocyanidins. The most well-known flavonols are quercetin and fisetin; flavones include luteolin, chrysin and baicalein; hesperetin belongs to the flavanones subclass; and genistein and daidzein are recognized as isoflavones (10). Numerous experimental studies have shown that these natural polyphenols exhibit various beneficial anti-cancer properties, such as antioxidant, anti-inflammatory,

Correspondence to: Katrin Sak, Clinic of Hematology and Oncology, University of Tartu, L. Puusepa 8, 51014 Tartu, Estonia. Tel: +372 53341381, e-mail: katrin.sak@ut.ee

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antiproliferative, pro-apoptotic, antiangiogenic as well as antimetastatic effects (10). However, the current knowledge about co-effects of flavonoids with cytotoxic chemotherapy is still rather poor. Therefore, the aim of this study was to thoroughly assess the cytotoxic activity of different flavonoids and their interactions with chemotherapeutics docetaxel and cabazitaxel in the human metastatic prostate cancer cell line PPC-1.

Materials and Methods

Cell line and flavonoids. In the current work, the cytotoxic activity of flavonoids and their effects on the efficacy of docetaxel and cabazitaxel in human metastatic prostate cancer cell line PPC-1 were studied. In experimental models, this cell line has been shown to be tumorigenic and highly metastatic, resembling the clinical situation of metastatic prostate cancer with visceral metastases requiring taxane-based systemic chemotherapy (11). Structures of the studied flavonoids are presented in Figure 1.

Chemicals. All flavonoids (baicalein, chrysin, fisetin, hesperetin, luteolin, quercetin, genistein and daidzein) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and L-glutamine were the products of Sigma-Aldrich (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) was from Mediatech, Inc (Manassas, VA, USA). Phosphate buffered saline (PBS) was obtained from Lonza (Verviers, Belgium). The chemotherapeutics, docetaxel (Accord, London, UK) and cabazitaxel (Sanofi-Aventis, Paris, France) were used.

Cell culture conditions. PPC-1 human prostate cancer cells were cultivated in Dulbecco's Modified Eagle's Medium (DMEM) (Life Technologies Corporation, Grand Island, NY, USA) supplemented with 10% of heat-inactivated fetal bovine serum (FBS) (Invitrogen™ , Auckland, NZ, USA). Cells were maintained in an incubator at 37˚C with 5% CO_2 and passaged 2-3 times per week.

Measurement of cell viability by MTT assay. The cytotoxic activities of taxanes and flavonoids in PPC-1 cells were tested by the MTT colorimetric assay, first described by Mosmann in 1983 (12). In detail, the cells were plated on to 96-well U-shaped bottom plates at a concentration of 1×10^5 cells/ml of medium, as a 100 μl of suspension. Cells were counted in a Bürker counting chamber. As phenol red can interfere with the reading of absorbance (13), the cells were seeded in phenol red-free RPMI-1640 medium (Mediatech, Inc, Manassas, VA, USA). After overnight incubation, cells were treated with varying doses of taxanes (0.01 nM-100 nM), flavonoids (10 nM-500 μM) or different combinations of taxanes and flavonoids for 48 h at 37˚C and 5% CO_2. At the end of the incubation, 50 μl of MTT solution in PBS was added to the wells in a final concentration of MTT of 5 mg/ml. Plates were further incubated for 4 h followed by centrifugation at 1,000 rpm for 10 min and removal of the supernatant. To dissolve the purple formazan crystals 150 μl of DMSO was added and the plates were shaken for 30 min. Absorbance was measured at 540 nm using a LED based microplate reader (Ledetect 96, Labexim Products, Austria). To calculate the proportion of surviving cells, the following formula was used: (OD of drug-treated sample – OD of blank)/(OD of control – OD of blank) ×100%, where OD of blank represents the absorbance reading of wells containing the buffer only (without cells) and OD of control represents the reading value of wells without any added test compounds. Dose-response curves were constructed to evaluate the half-maximal inhibitory concentrations (IC_{50} values). All experiments were performed in triplicates.

Statistics. Data were analyzed using the GraphPad Prism statistical software. Kolmogorov–Smirnov test for normality was performed to evaluate if the data were in normal distribution. The one-way analysis of variance (ANOVA) was applied to determine whether there were any significant differences between the means. *p*-Values less than 0.05 were considered as statistically significant and all values were expressed as mean±standard deviation (SD).

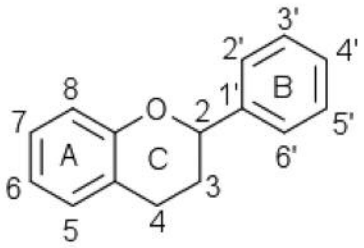
Results

Cytotoxic profiles of taxanes and flavonoids in PPC-1 human prostate cancer cells. Both taxanes, docetaxel and cabazitaxel, suppressed the viability of PPC-1 cells with the half-maximal inhibitory constants (IC_{50}) of 1.7±0.6 nM for docetaxel and 0.5±0.2 nM for cabazitaxel (Figure 2a, Table I). Tested flavonoids exerted cytotoxic activity only at high micromolar concentrations with IC_{50} values of 78.5±1.3 μM for quercetin, 80.2±1.5 μM for fisetin, 83.6±1.4 μM for luteolin and 103.8±1.4 μM for baicalein, or revealed no remarkable effect on cell survival with concentrations up to 100 μM (chrysin, hesperetin, genistein, daidzein) (Figure 2b, Table I).

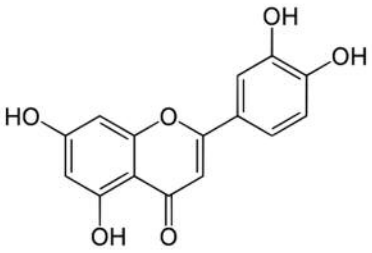
Effect of flavonoids on cytotoxicity of docetaxel and cabazitaxel in PPC-1 human prostate cancer cells. Simultaneous treatment of PPC-1 cells with taxanes and flavonoids (baicalein, chrysin, luteolin, fisetin, quercetin, genistein or daidzein) at 10 μM concentration did not lead to any shift in the dose response curves of docetaxel or cabazitaxel showing no potentiating or suppressive effect of these plant secondary metabolites (data not shown).

Simultaneously added hesperetin decreases cytotoxicity of docetaxel and cabazitaxel in PPC-1 human prostate cancer cells. In contrast to all other tested flavonoids, simultaneous exposure of cells to 10 μM hesperetin and taxanes resulted in about three-fold decrease in cytotoxic, *i.e.* three-fold increase in IC_{50} values, of both chemotherapeutics. Moreover, 30 μM of hesperetin resulted in 9.8- and 13.1-fold reduction in the cytotoxicity of docetaxel and cabazitaxel, respectively (Figure 3a and 3b, Table II). When cells were cotreated with cabazitaxel and 30 μM hesperetin, this inhibitory effect reached statistical significance (p=0.04) (Table II).

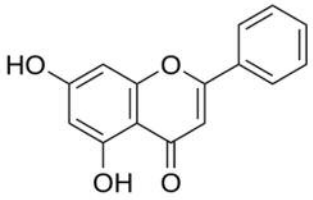
Hesperetin did not interfere with taxane-induced cytotoxicity in PPC-1 cells when added 24 h after taxane treatment. If 30 μM hesperetin were added 24 h after treatment of cells with docetaxel or cabazitaxel, no suppressive effect of this citrus flavonoid on taxanes cytotoxicity was observed (Figure 4a and 4b).



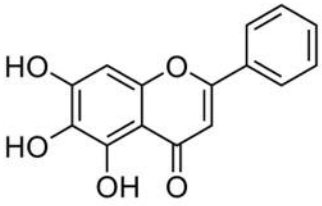
General skeleton of flavonoids



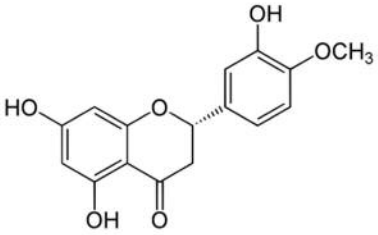
Luteolin



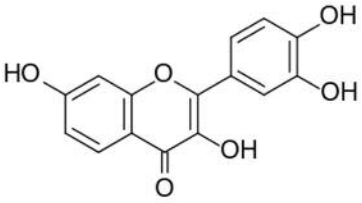
Chrysin



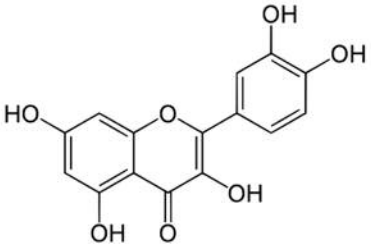
Baicalein



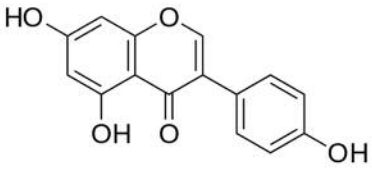
Hesperetin



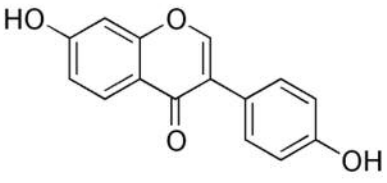
Fisetin



Quercetin



Genistein



Daidzein

Figure 1. *Chemical structures of tested flavonoids*.

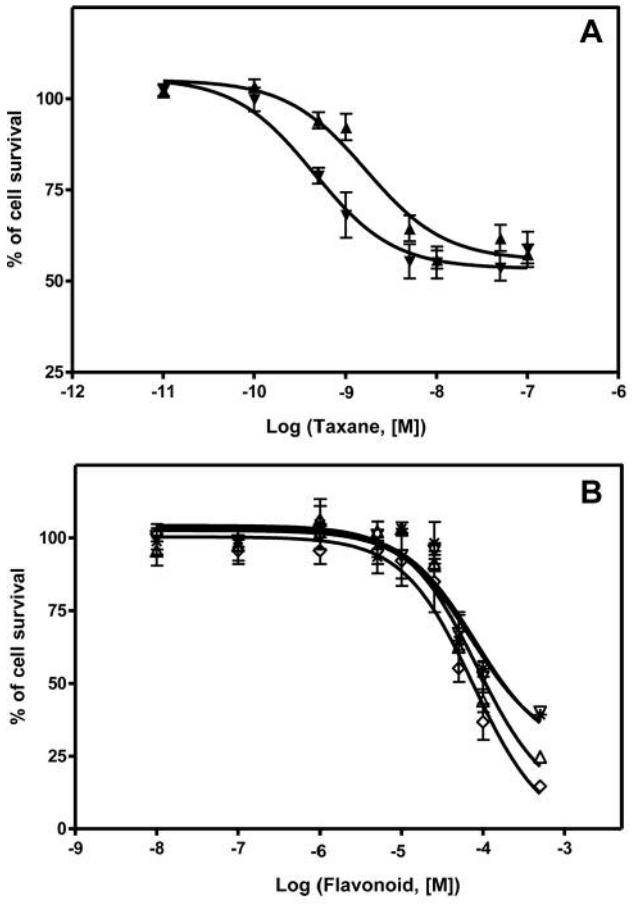


Figure 2. *Cell growth inhibitory effects of taxanes (docetaxel ▲, cabazitaxel ▼; A) and cytotoxically active flavonoids (luteolin △, baicalein ▽, quercetin ◇, fisetin *; B) in PPC-1 human prostate cancer cells by treating the cells with respective compounds for 48 h.*

Table I. *IC_{50} values of taxanes and flavonoids in PPC-1 human prostate cancer cells following 48 h of treatment (data are expressed as mean values±standard deviation (SD)).*

Chemotherapeutics	IC_{50}, nM
Taxanes	
Docetaxel	1.7±0.6
Cabazitaxel	0.5±0.2
Flavonoids	**IC_{50}, μM**
Flavones	
Baicalein	103.8±1.4
Chrysin	197.2±1.4
Luteolin	83.6±1.4
Flavanones	
Hesperetin	268.5±1.6
Flavonols	
Fisetin	80.2±1.5
Quercetin	78.5±1.3
Isoflavones	
Genistein	>>100
Daidzein	>>100

Table II. *Antagonistic effect of 10 μM and 30 μM hesperetin on docetaxel or cabazitaxel cytotoxicity in PPC-1 human prostate cancer cells by combined treatment of cells for 48 h. Statistically significant difference ($p<0.05$) is marked with an asterisk.*

	Ratio of IC_{50} for taxane in the presence of hesperetin and IC_{50} for taxane alone	*p*-Value
Docetaxel	1.0	
+10 μM hesperetin	3.0	0.62
+30 μM hesperetin	9.8	0.13
Cabazitaxel	1.0	
+10 μM hesperetin	2.8	0.56
+30 μM hesperetin	13.1	0.04*

Discussion

More than half of prostate cancer patients use additionally some kind of CM product during the course of active anti-cancer therapy. However, interactions of CM with conventional anticancer therapies, including taxane-based chemotherapy, are largely unknown. Therefore, we assessed the cytotoxic activity of different flavonoids and their interactions with the chemotherapeutics docetaxel and cabazitaxel in human metastatic prostate cancer cell line PPC-1.

Both taxanes, docetaxel and cabazitaxel, significantly decreased the viability of PPC-1 cells as measured by MTT assay. Sensitivity to docetaxel has been described earlier (14); however, no study was identified on the cytotoxic effect of cabazitaxel in PPC-1 cell line was identified. Next to taxanes, the cytotoxic profile of the number of commercially available flavonoids was examined. Our study showed that some flavonoids exerted cytotoxic activity only at high micromolar concentrations (quercetin, fisetin, luteolin, baicalein) while other flavonoids had no significant effect on cell survival (chrysin, hesperetin, genistein, daidzein). Although similar cytotoxic effects have been seen in PPC-1 cell line for quercetin, there are no reports describing the effects of the other tested flavonoids (15). Few studies have described the cytotoxic actions of flavonoids such as luteolin, genistein and chrysin but using other prostate cancer cell lines (*e.g.*, LNCaP, PC3, DU145) (16-20).

To further characterize the effects of flavonoids, their combinations with the chemotherapeutics docetaxel or cabazitaxel were investigated. Simultaneous treatment of

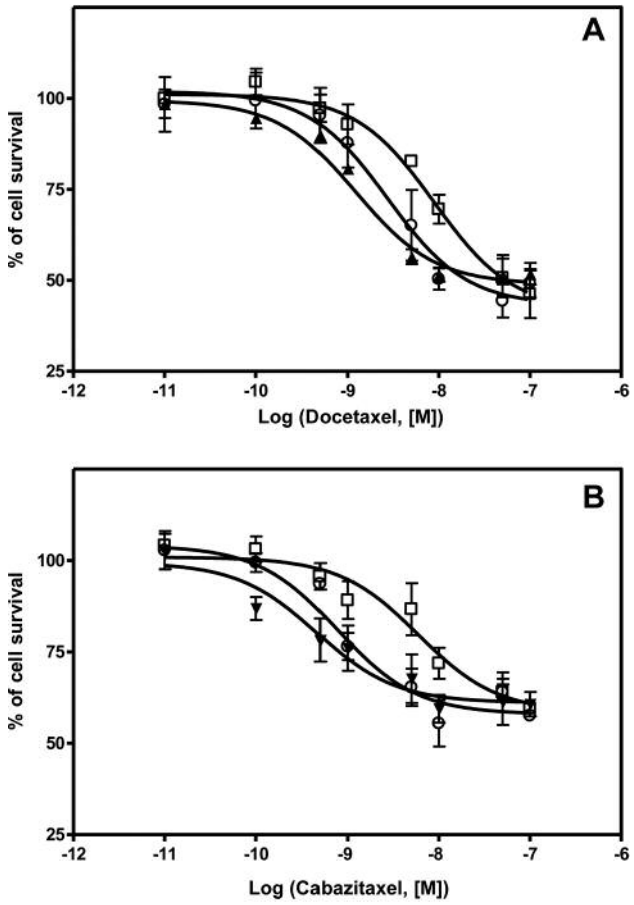


Figure 3. *Suppression of docetaxel (▲, A) and cabazitaxel (▼, B) cytotoxicity by 10 μM (○) and 30 μM (□) hesperetin in PPC-1 human prostate cancer cells by treating the cells with flavonoids and taxanes simultaneously for 48 h.*

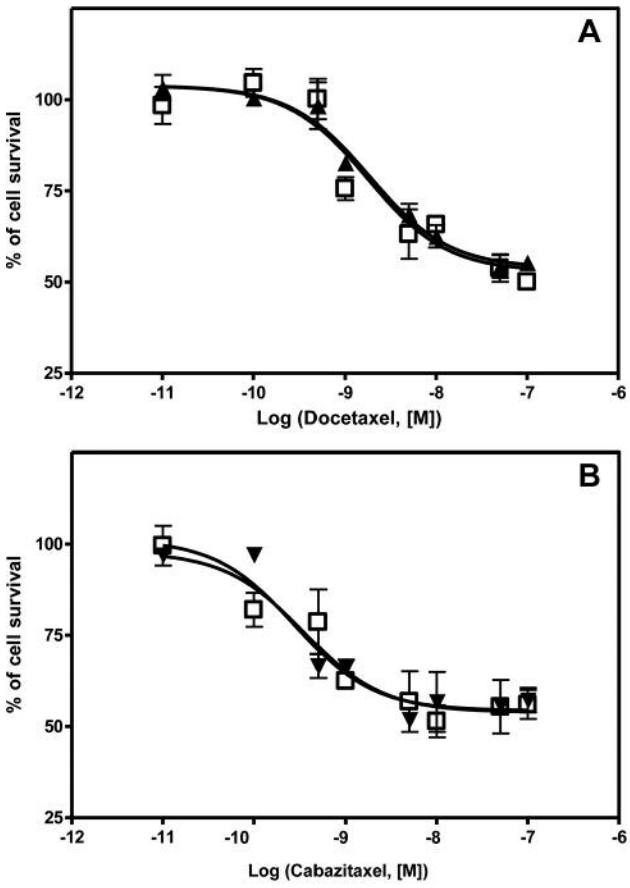


Figure 4. *Treatment of PPC-1 human prostate cancer cells pretreated with docetaxel (▲, A) or cabazitaxel (▼, B) for 24 h with 30 μM hesperetin (□) had no effect on taxane-induced cytotoxicity.*

PPC-1 cells with taxanes and flavonoids (baicalein, chrysin, luteolin, fisetin, quercetin, genistein or daidzein) did not lead to any shift in the dose response curves of docetaxel or cabazitaxel showing no potentiating or suppressive effect of these plant secondary metabolites. This is in contrast to other prostate cancer cell lines where a potentiating effect of some flavonoids on taxane cytotoxicity has been reported. For example, in the case of human prostate cancer cells 22Rv1, PC-3M-luc-6 or C4-2, the flavonol fisetin was shown to enhance the cytotoxic effects of cabazitaxel (21). Similar sensitization of PC-3, C4-2 or $ARCaP_M$ cells to cabazitaxel response was observed also in combination with the isoflavone genistein (22). Moreover, the therapeutic efficacy of docetaxel was enhanced by the presence of quercetin in PC-3-engineered xenograft prostate tumors (23). These differences suggest that the behavior of flavonoids largely depends on their structure as well as the specific features of prostate cancer cell line, probably including their distinct hormone and taxane sensitivity.

The most important finding of this study was that one of the tested flavonoids – citrus flavanone hesperetin – decreased the effect of taxanes. Both docetaxel and especially cabazitaxel cytotoxicity was markedly reduced by simultaneous addition of hesperetin to culture media. Interestingly, this inhibitory effect of hesperetin was only seen upon simultaneous treatment with taxane and not when prostate cancer cells were treated with the flavonoid 24 h after treatment with the chemotherapeutic and flavonoid administration, showing that only concomitant co-effects are important.

It has been previously shown that flavonoids may participate in different intermolecular and intercellular interactions. Flavonoids are able to penetrate into biological membranes, particularly in compartments known as lipid rafts. By influencing physical properties of the lipid bilayer, flavonoids may control the arrangement of membrane proteins and the formation of functional complexes responsible for cellular signal transduction and the regulation

of the metabolism (24). For example, it has been reported that hesperetin improves antioxidant status and membrane lipid compositions in rat liver (25). Moreover, in Caco-2 intestinal epithelial cell monolayers, hesperetin has been shown to increase transepithelial electrical resistance and the expression of tight junction proteins such as occludin and claudins 1, 3 and 4 (26). The latter shows that hesperetin influences cell membrane barrier integrity and therefore it is rather plausible that by increasing the expression of tight junction proteins, it may affect entry of chemotherapeutics into the cancer cells and thereby reduce the cytotoxic effects of anti-cancer therapy.

There are only few studies that have examined anti-cancer treatment combinations with hesperetin. In an *in vivo* study in rats, oral administration of hesperetin in combination with doxorubicin suppressed the expression of some apoptosis-associated genes, such as NF-κB, p38 and caspase-3 (27). However, in combination with hormonal therapy (bicalutamide), hesperetin has been shown to potentiate apoptotic effects of cancer therapy in taxane-resistant PC-3 prostate cancer cells (28), pointing again toward the possibility that the effect of flavonoids on prostate cancer cells may be different in castration- and taxane- sensitive and castration- and taxane-resistant clinical situations.

Although the action of natural products in cancer patients can be rather different from experimental laboratory systems due to side-effects and safety (29), but also bioavailability, *i.e.*, intestinal absorption, metabolic conversion and excretion of these compounds from the human body (30), there is no doubt that caution is needed when consuming plant-derived hesperetin supplements during the active taxane-based chemotherapy of advanced prostate cancer. Also, caution is needed in all dietary recommendations, since hesperetin can be found abundantly in various citrus fruits, such as oranges, mandarins, tangerines, limes, lemons, pomelos and grapefruits (30, 31). Additionally, it is important to know that another important source of this flavanone includes the herb peppermint (30).

Conclusion

Our study showed that dietary flavonoid hesperetin remarkably decreased the cytotoxic effect of chemotherapeutics docetaxel and especially cabazitaxel in prostate cancer cells. Therefore, caution is clearly needed for prostate cancer patients who take oral supplements containing hesperetin or follow special diets rich in citrus fruits and peppermint.

Conflicts of Interest

The Authors declare that there is no conflict of interest regarding the publication of this paper.

Acknowledgements

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