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INFLUENCE OF COMMON TANSY (*Tanacetum vulgare*) FLOWER POLYSACCHARIDE COMPLEX ON P-GLYCOPROTEIN TRANSPORTER ACTIVITY *IN VITRO*

I. V. Chernykh,[1,*] A. V. Shchul'kin,[1] E. E. Kirichenko,[1] A. S. Esenina,[1] M. M. Gradinar',[1] A. A. Slepnev,[1] and E. N. Yakusheva[1]

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The influence of the common tansy (*Tanacetum vulgare*) flower polysaccharide complex on P-glycoprotein (Pgp, ABCB1-protein) activity in Caco-2 cell line was studied. The *in vitro* Pgp activity was assessed by measuring fexofenadine transport in a transwell-system. The ratio of apparent permeability coefficients *b–a*/*a–b* for fexofenadine decreased by 57.1, 62.7, and 72.1% as compared to a control series if the transport medium was treated with the polysaccharide complex at concentrations of 7, 10, and 100 μM, respectively. This indicated that the polysaccharide decreased the ABCB1-protein activity. A comparison of the apparent permeability coefficients of fexofenadine and their ratio for the Pgp inhibitor verapamil and the polysaccharide complex from common tansy flowers showed that the polysaccharide inhibited Pgp more than verapamil. The IC_{50} of the studied polysaccharide complex was calculated and allowed the dose for *in vivo* studies to be determined.

Keywords: P-glycoprotein, inhibitors, plant-derived polysaccharides, common tansy, Caco-2 cell line.

P-glycoprotein (Pgp, ABCB1-protein) is an ATP-dependent transporter protein, the increased activity of which is a key factor in the resistance of tumor cells to chemotherapy [1] and the development of pharmacoresistant epilepsy [2]. Pgp is nonspecific for substrate, making it responsible for the efflux of a broad spectrum of compounds from cells and rendering them unreceptive to administered pharmacotherapy. Thus, this transporter protein should be pharmacologically inhibited to increase the efficacy of medicines (increase drug penetration into cells).

Until now, plant-derived preparations have not been proposed as ABCB1-protein inhibitors despite their insignificant side effects and economic availability as compared to synthetic agents [3, 4]. Plant-derived polysaccharides are used in medical practice mainly as herbal preparations and are considered promising pharmaceuticals. They possess several important advantages, e.g., susceptibility to various chemical modifications, biocompatibility, biodegradability, and low immunogenicity [5].

[1] I. P. Pavlov Ryazan State Medical University, Ministry of Health of the Russian Federation, Ryazan, 390026 Russia.
[*] e-mail: ivchernykh88@mail.ru

The goal of the research was to study the effect of the common tansy (*Tanacetum vulgare*) flower polysaccharide complex on the *in vitro* activity of Pgp transporter.

EXPERIMENTAL PART

The polysaccharide complex was isolated from air-dried common tansy flower raw material (Fitofarm, Russia) using the previously reported method [6]. Then, acid hydrolysis of the obtained substance using H_2SO_4 (0.1 M) for 3 h produced a polysaccharide complex of molecular mass 3.8 kDa. Impurities of free mono- and disaccharides were determined by normal-phase HPLC over a Luna NH2 column (250 × 4.6 mm, 5 μm) using mobile phase MeCN–H_2O (70:30) and refractive-index detection at room temperature [7]. The contents of unbound fructose (0.09%) and sucrose (0.49%) were recalculated for dry substance. The polysaccharide was standardized via titration with alkali to give the number of free carboxylic acids and by spectrophotometry to give the content of reducing monosaccharides (recalculated as glucose) after total acid hydrolysis of the substance by picric acid [8]. This reaction was stoichiometric with a sensi-

tivity of 0.03 mg/L calculated as glucose so that the utilized standardization method was more accurate and faster than the pharmacopoeial gravimetric method [9].

The effect of the common pansy flower polysaccharide complex on ABCB1-protein activity was studied using Caco-2 (human epithelial colorectal adenocarcinoma) cells with hyperexpression of ABCB1-protein (Inst. Cytol., RAS, St. Petersburg).

Cells were cultivated at 37°C and 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM) with a high glucose content (4,500 mg/L), L-glutamine (4 mM), bovine serum (15%), penicillin (100 U/mL) and streptomycin (100 μg/mL). Cells were re-inoculated onto semipermeable membranes of a transwell-system wells after reaching 70 – 90% confluency (Fig. 1).

Cells in transwell-system wells were cultivated for 21 d in the same growth medium to attain transepithelial resistance >500 mΩ·cm^2.

The activity of Pgp *in vitro* was assessed from the transport of fexofenadine tracer substrate in the transwell-system. For this, growth medium was replaced by Hank's solution with HEPES buffer (25 mM, pH 7.4) and DMSO (1%, transport medium). First, a blank study was conducted by adding fexofenadine (Sigma, USA) to the basal-lateral chamber to a final concentration of 150 μM [10]. Aliquots (50 μL) of the apical chamber medium were taken at 1, 2, and 3 h to determine the concentration of tracer substrate (*b–a* transport due to passive diffusion and Pgp efflux activity). Fexofenadine transport in another transwell-system was assessed from the apical into the basal-lateral chamber (*a–b* transport due to passive diffusion opposing the action of ABCB1-protein). For this, tracer substrate (150 μM) was added to the apical chamber. Aliquots (50 μL) were taken after 1, 2, and 3 h from the basal-lateral chamber to determine the fexofenadine concentration.

Fexofenadine transport both from chamber *a* into chamber *b* and in the opposite direction was assessed using the formula [11]:

$$P_{app} = \frac{dQ}{dt} \cdot \frac{1}{(A \cdot C_0)},$$

where P_{app} is the apparent permeability coefficient; dQ/dt, change of substrate concentration in the receiving chamber over the incubation time; A, area of the well semi-permeable membrane in the transwell-system in which the cells were cultivated; and C_0, initial substrate concentration in the donating chamber.

Next, the ratio of apparent permeability coefficients *b–a* to *a–b* was calculated:

$$\text{Ratio of coefficients} = \frac{P_{app}\, b-a}{P_{app}\, a-b}.$$

This was an integral parameter and assessed the total contribution of Pgp to fexofenadine transport through the lipid bilayer.

The next stage of the research studied the transport of ABCB1-protein tracer substrate with the classical Pgp inhibitor verapamil (Sigma, USA) added to the apical and basal-lateral chambers at concentrations of 1, 10, 50, 100, and 200 μM [12]. Then, transport of fexofenadine in the presence of the common pansy flower polysaccharide complex at concentrations of 1, 3, 7, 10, and 100 μM was studied.

The IC_{50} values (concentration of half-maximum inhibition) for the tested compounds were calculated using the results [13].

Each series of experiments was performed in triplicate.

The fexofenadine concentration in the transport medium was determined by HPLC with UV detection at 220 nm on a Staier HPLC (Russia) using an in-house method.

The obtained aliquot of transport medium (50 μL) containing fexofenadine was diluted with mobile phase (150 μL). The resulting solution (100 μL) was injected into the chromatograph. The analyses used a Phenomenex Synergi 4-μm Polar-RP 80A chromatography column (250 × 4.6 mm, 4 μm) at 35°C and flow rate 1 mL/min. The mobile phase was MeCN (128 mL), deionized H_2O (267.4 mL), glacial HOAc (6.33 mL), and Et_3N to pH 6.7. The retention time of fexofenadine under these conditions was 12.8 min. Quantitative determination used peak areas for absolute calibration. The analytical range of the method was 1.2 – 57.4 μM.

The results were processed using the Statsoft Statistica 8.0 program. The statistical significance of the results was assessed using dispersion analysis. Pairs were compared using the Newman–Keuls criterion. Differences were considered statistically significant for $p < 0.05$.

RESULTS AND DISCUSSION

ABCB1-protein activity *in vitro* was assessed from the transport of fexofenadine transporter protein tracer substrate in the transwell-system with Caco-2 cells.

First, fexofenadine transport from the basal-lateral into the apical chamber (*b–a* transport) was studied in control experiments. This parameter characterized transport of the substances through the Caco-2 cell lipid bilayer due to passive diffusion and active transport using specific transporters. The apparent permeability coefficient *b–a* of fexofenadine was $(3.79 \pm 0.34) \cdot 10^{-6}$ cm/s.

Next, transport of fexofenadine from the apical into the basal-lateral chamber (*a–b* transport) was assessed. It characterized transport of the substances through the Caco-2 cell lipid bilayer due only to passive diffusion opposing the ac-

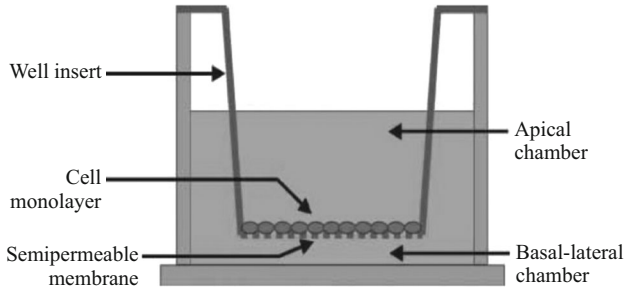

Fig. 1. Schematic diagram of the transwell-system.

tion of Pgp. The apparent permeability coefficient *a–b* of fexofenadine was $(0.64 \pm 0.21) \cdot 10^{-6}$ cm/s.

Finally, the ratio of fexofenadine apparent permeability coefficients *b–a* and *a–b* was calculated. This parameter indicated if specific transporters (ABCB1-protein is a specific transporter for fexofenadine) were involved in transport of the tested substance. Specific transporters were considered to be involved in transporting the substance if the ratio of coefficients was greater than two. Otherwise, transport was primarily due to passive diffusion.

The ratio of apparent permeability coefficients for fexofenadine was 6.27 ± 1.70, which was greater than two and confirmed that Pgp was involved in fexofenadine transport (Table 1).

Verapamil, a classical ABCB1-protein inhibitor, at final concentrations of 1 and 10 μM in the transport medium did not statistically significantly affect fexofenadine apparent permeability coefficients *b–a* and *a–b* and their ratio as compared to the control values.

Verapamil concentrations of 50, 100, and 200 μM in the transport medium reduced fexofenadine apparent permeability coefficient *b–a* by 61.2% ($p < 0.05$), 69.7% ($p < 0.05$), and 67.5% ($p < 0.05$), respectively, as compared to the control values. However, fexofenadine apparent permeability coefficient *a–b* did not change. The ratio of coefficients *b–a*/*a–b* diminished by 55.2% ($p < 0.05$), 62.2% ($p < 0.05$), and 72.7% ($p < 0.05$) as compared to the corresponding control values.

The common pansy flower polysaccharide complex at concentrations of 1 and 3 μM did not affect fexofenadine apparent permeability coefficients *b–a* and *a–b* and their ratio.

However, this complex at a concentration of 7 μM increased fexofenadine apparent permeability coefficient *a–b* by 206.3% ($p < 0.05$) and reduced the ratio of coefficients *b–a*/*a–b* by 57.1% ($p < 0.05$) as compared to the control. The complex at a concentration of 10 μM reduced apparent permeability coefficient *b–a* by 30.1% ($p < 0.05$) and the ratio of coefficients *b–a*/*a–b* by 62.7% ($p < 0.05$) and increased apparent permeability coefficient *a–b* by 75.0% ($p < 0.05$). The complex at a concentration of 100 μM decreased apparent permeability coefficient *b–a* by 48.5% ($p < 0.05$) and ratio of coefficients *b–a*/*a–b* by 72.1% ($p < 0.05$) and increased apparent permeability coefficient *a–b* by 73.4% ($p < 0.05$).

A comparison of fexofenadine apparent permeability coefficients with added verapamil and polysaccharide found that coefficient *b–a* and ratio *b–a*/*a–b* with polysaccharide at a concentration of 10 μM were statistically significantly less than those of a series with verapamil at this same concentration by 31.3 ($p < 0.05$) and 45.1% ($p < 0.05$), respectively.

The IC_{50} value for Pgp was 24.5 ± 14.17 μM for verapamil and 8.04 ± 2.6 μM for the studied polysaccharide complex.

The chemical structures were responsible for the ability to use plant-derived polysaccharides as ABCB1-protein inhibitors because they contained functional groups characteristic of transporter inhibitors, i.e., they contained highly

TABLE 1. Transport of Fexofenadine (150 μM) Through Caco-2 Cell Line Lipid Bilayer Membrane

	P_{app} *b-a*	P_{app} *a-b*	P_{app} *b-a*/P_{app} *a-b*
Control	$3.79 \cdot 10^{-6} \pm 0.34 \cdot 10^{-6}$	$0.64 \cdot 10^{-6} \pm 0.21 \cdot 10^{-6}$	6.27 ± 1.70
Verapamil 1 μM	$3.55 \cdot 10^{-6} \pm 1.09 \cdot 10^{-6}$	$0.76 \cdot 10^{-6} \pm 0.26 \cdot 10^{-6}$	4.78 ± 0.44
Verapamil 10 μM	$3.86 \cdot 10^{-6} \pm 0.20 \cdot 10^{-6}$	$0.92 \cdot 10^{-6} \pm 0.14 \cdot 10^{-6}$	4.26 ± 0.44
Verapamil 50 μM	$1.47 \cdot 10^{-6} \pm 0.25 \cdot 10^{-6}$*	$0.55 \cdot 10^{-6} \pm 0.19 \cdot 10^{-6}$	$2.81 \pm 0.69^{*}$
Verapamil 100 μM	$1.15 \cdot 10^{-6} \pm 0.40 \cdot 10^{-6*}$	$0.51 \cdot 10^{-6} \pm 0.156 \cdot 10^{-6}$	$2.37 \pm 0.83^{*}$
Verapamil 200 μM	$1.23 \cdot 10^{-6} \pm 0.19 \cdot 10^{-6*}$	$0.85 \cdot 10^{-6} \pm 0.45 \cdot 10^{-6}$	$1.71 \pm 0.82^{*}$
Polysaccharide 1 μM	$4.53 \cdot 10^{-6} \pm 0.83 \cdot 10^{-6}$	$0.54 \cdot 10^{-6} \pm 0.24 \cdot 10^{-6}$	9.12 ± 2.4
Polysaccharide 3 μM	$3.28 \cdot 10^{-6} \pm 0.24 \cdot 10^{-6}$	$0.51 \cdot 10^{-6} \pm 0.06 \cdot 10^{-6}$	6.42 ± 0.94
Polysaccharide 7 μM	$4.88 \cdot 10^{-6} \pm 0.86 \cdot 10^{-6}$	$1.96 \cdot 10^{-6} \pm 0.61 \cdot 10^{-6*}$	$2.69 \pm 0.98^{*}$
Polysaccharide 10 μM	$2.65 \cdot 10^{-6} \pm 0.62 \cdot 10^{-6*,\#}$	$1.12 \cdot 10^{-6} \pm 0.08 \cdot 10^{-6*}$	$2.34 \pm 0.39^{*,\#}$
Polysaccharide 100 μM	$1.95 \cdot 10^{-6} \pm 0.76 \cdot 10^{-6*}$	$1.11 \cdot 10^{-6} \pm 0.03 \cdot 10^{-6*}$	$1.75 \pm 0.68^{*}$

* $p < 0.05$ are statistically significant differences vs. the control; # $p < 0.05$, vs. the verapamil series of the corresponding concentration.

electronegative O atoms that could supply electron pairs for forming both intramolecular H-bonds and bonds to Pgp [14].

Several poly- and oligosaccharides were shown in previous research to be ABCB1-protein substrates. Heparin is a negatively charged highly sulfated polysaccharide that could increase intracellular accumulation of drug preparations and Pgp substrates in tumor-cell cultures by functional inhibition of several transporters in the adenosine binding cassette (ABC) family. This may have been due to the ability of heparin to bind many extracellular proteins (growth factors, extracellular matrix components) and modulate their activity because of its charge [15]. Analogous results were obtained in *in vitro* studies using human breast tumor cell culture (MDA-MB231). Unfractionated heparin was found to inhibit Pgp functioning during analyses of intracellular penetration of fluorescent transporter substrate calcein acetoxymethyl ester [16].

Several resinous glycosides (glycolipids or lipooligosaccharides) from seeds of *Ipomoea alba* increased the uptake of vinblastine by human breast carcinoma culture with multiple drug resistance [1].

Cell line Caco-2 is widely used to study drug transport by ABCB1-protein and the effects of various substances on transporter activity [11]. The morphology and functioning of this cell line are characteristically much like those of human intestinal (absorbing) enterocytes [17] in which Pgp is normally expressed.

Transport of ABCB1-protein tracer substrate fexofenadine from the basal-lateral into the apical chamber after adding verapamil, a compound with proven ability to inhibit Pgp [9], at concentrations of 50, 100, and 200 μM was suppressed. Also, the ratio of apparent permeability coefficients *b–a*/*a–b* decreased as compared to the control experiments. Verapamil added to the transwell-system at concentrations of 1 and 10 μM did not change coefficients *b–a* and *a–b* and their ratio. Thus, verapamil at these concentrations did not affect transporter-protein activity.

The ratio of apparent permeability coefficients *b–a*/*a–b* decreased after adding the common tansy flower polysaccharide complex at concentrations of 7, 10, and 100 μM to the transport medium with *b–a* decreasing (10 and 100 μM) and *a–b* increasing (7, 10, and 100 μM). The results indicated that the polysaccharide inhibited Pgp functioning. The polysaccharide complex at lower concentrations did not cause such changes.

The lower *b–a* values and *b–a*/*a–b* ratios for the series using the polysaccharide at a concentration of 10 μM as compared to the verapamil series at an analogous concentration indicated that the polysaccharide inhibited ABCB1-protein more than verapamil. The fact that IC_{50} for verapamil was three times greater than that for the polysaccharide complex also confirmed this.

It is noteworthy that the *in vitro* testing could detect only direct inhibitors of ABCB1-protein, i.e., compounds that reduced transporter activity through molecular interactions. Several substrate-binding sites and sites regulating transporter functioning were found in the Pgp molecule [18]. Thus, the common tansy flower polysaccharide complex probably reduced directly the transporter activity.

The IC_{50} value calculated by us for the common tansy flower polysaccharide complex allowed the polysaccharide dose for studying its effects on ABCB1-protein in *in vivo* experiments to be determined and the potential of this substance as an antitumor or antiepileptic agent to be analyzed.

Thus, the common tansy (*T. vulgare*) flower polysaccharide complex in *in vitro* experiments inhibited ABCB1-protein functioning more than the classical transporter inhibitor verapamil.

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