

ABSORPTION AND METABOLISM OF BIOLOGICALLY ACTIVE GENISTEIN DERIVATIVES IN COLON CARCINOMA CELL LINE (CACO-2)

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Abstract: Several genistein derivatives comprising an isoflavonoid skeleton substituted with an alkyl chain and a sugar moiety show ability to inhibit proliferation of cancer cells *in vitro* at the concentration several-fold lower than genistein. In our previous studies we shown that these compounds influenced the mitotic spindle, blocked the cell cycle and induced apoptosis. The purpose of this study was to determine the relationship between structural modifications of genistein molecule and the intestinal disposition of its derivatives. Transport and metabolism of these compounds were studied in the human intestinal Caco-2 model. The results of our study indicate that transport and metabolism of genistein derivatives depend both, on the structure of the carbonyl linker and position of genistein molecule substitution. All new compounds showed higher permeability coefficient in comparison to genistein. Moreover, genistein derivatives described in this work were transformed in Caco-2 cells into glucuronide and sulfate metabolites.

Keywords: genistein, metabolism, Caco-2, LC-MS/MS

Genistein (5,7-dihydroxy-3-(4-hydroxyphenyl)chromen-4-one) is considered as a useful compound for prophylaxis or treatment of many pathological conditions, such as cancer, menopausal syndrome, cardiovascular disease, osteoporosis and diabetes. The most promising applications of this isoflavonoid are associated with cancer chemoprevention. Many *in vitro* and *in vivo* studies demonstrating antiproliferative activity of genistein go along with epidemiological data, linking genistein rich diet with reduced risk of some types of cancer. Genistein inhibits proliferation of tumor cells due to inhibition of activity of several molecular targets, including transcriptional factor NF- κ B, tyrosine kinases and topoisomerase II (1–4).

However, despite many beneficial properties, the use of this compound *in vivo* is limited due to weak solubility in water, fast biotransformation to inactive metabolites, poor accumulation in tissues and target cells, and low concentration in blood after administration *per os* (5). In order to improve pharmacological properties and antitumor potency of genistein, new

derivatives were designed and synthesized by different laboratories (6). Very promising class of compounds, synthesized on the base of genistein, that exhibit anti-cancer potential are glycoconjugates and 2,3 unsaturated mono- or disaccharides of this isoflavonoid, described in our previous work. Our screening studies revealed several glycoconjugates of genistein inhibiting cancer cell proliferation at the concentration several fold lower than the parent compound. Depending on the structure of a sugar moiety and the type of substitution, new glycoconjugates of genistein inhibited cancer cell proliferation through different molecular mechanisms (7, 8). Some of them acted in a different manner than genistein and targeted mitotic spindles.

Next, we decided to correlate structural features of genistein derivatives with their bioavailability. To address this question, we decided to use Caco-2 model, regarded as an *in vitro* surrogate for studying intestinal absorption. This paper presents *in vitro* bioavailability parameters of genistein derivatives and discusses correlation between structural features and bioavailability of compounds.

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EXPERIMENTAL

Synthesis of genistein derivatives

Synthesis of tested compounds (Fig. 1) was described previously in (7–9).

Determination of absorption

General

Cancer cell line

Human Caucasian colon adenocarcinoma cell line (Caco-2) was obtained from ECACC (European Collection of Cell Cultures, UK). Cells were cultured in Dulbecco's modified Eagle medium – high glucose 4.5 g/L (Sigma Aldrich, USA), supplemented with 20% (v/v) inactivated fetal bovine serum [iFBS] (PAA Laboratories GmbH, Austria) and 1% (v/v) non-essential amino acids (Biological Industries, Israel). Additionally, gentamycin (KRKA, Slovenia) was added to culture media at the concentration 0.04 mg/mL. Cells were cultured at 37°C in humidified atmosphere of 5% CO₂.

MTT assay

Toxicity of tested compounds in Caco-2 cell line was estimated using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma Aldrich, USA) assay.

Caco-2 cells were plated in 96-well plates (Thermo Fisher Scientific, Nunc, Denmark) at the density of 6×10^4 cells per cm² and grown for 72 h. Then, the medium was aspirated and cells were treated for 24 h with the solution of tested compounds in growth medium. In the experiment, we used a series of concentrations: 5, 10, 25 and 50 μM. After 24 h, medium was removed and cells were incubated with 0.5 mg/mL MTT solution (50 μL) in Dulbecco's modified Eagle medium without phenol red (Sigma Aldrich, USA) for 3 h. Then, medium was aspirated and crystals of formazan were solubilized in 0.04 M solution of HCl in 2-propanol (Avantor Performance Materials, Poland). The absorbance of samples was measured spectrophotometrically with a microplate reader BioTek Synergy II (BioTek Instruments, USA) at 570 nm wavelength.

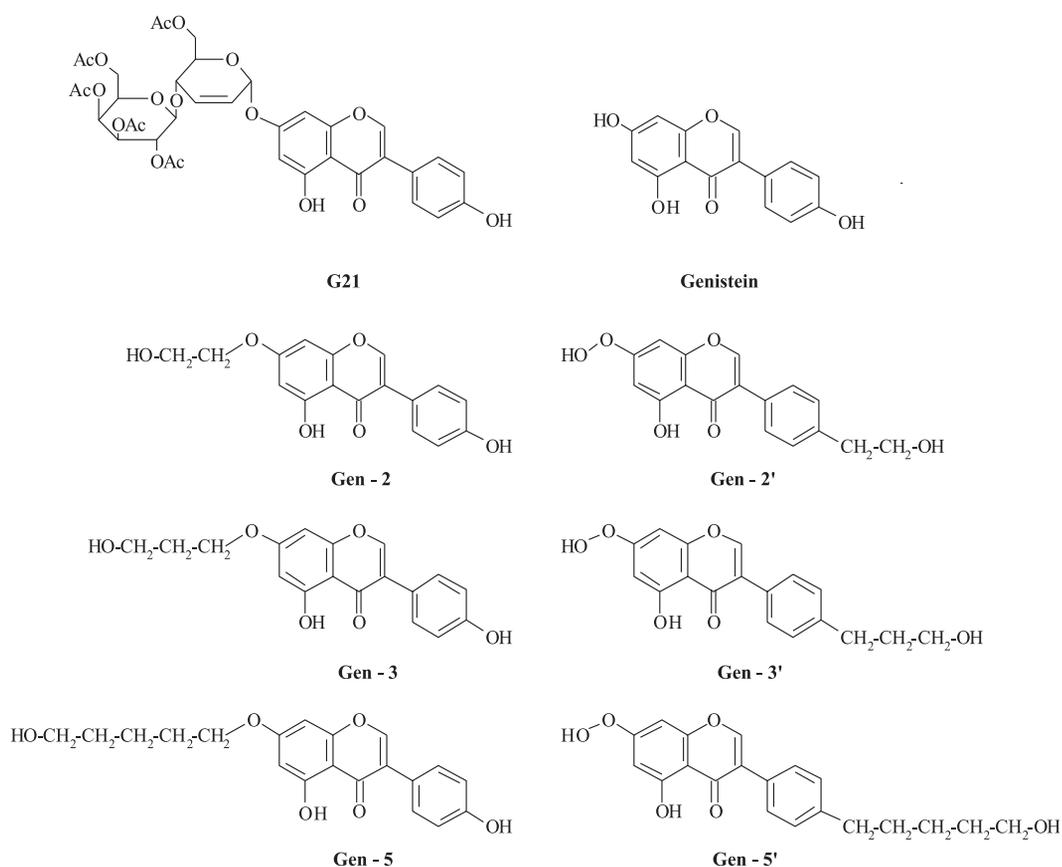


Figure 1. Structure of the tested compounds

Moreover, for two concentrations of compounds (25 and 50 μM), the additional experimental variant was performed, using Hanks' Balanced Salt (Sigma Aldrich, USA) solution with calcium and magnesium instead of MEM for preparation of compounds solution.

For each compound in a single experiment, every concentration was tested in quadruplicate. Experiments were repeated at least three times. The percentage of cell viability was calculated using the following equation:

% of cell viability in relation to untreated control

$$= \left(\frac{A - B}{C - B} \right) \cdot 100\%$$

where A = average of absorbance measured for wells with cells treated with a tested compound, B = average of absorbance measured for wells without cells (blank) C = average of absorbance for wells with cells non-treated cells (control).

Transport experiments

The experiment was based on protocols described in (10).

In the first step, Caco-2 cell line was cultivated on 24-well plates with porous membranes (Millicell PCF, 0.4 μm pore size) (Merck Millipore, Germany). The apical volume was 0.4 mL and basolateral volume was 0.6 mL. The cells were seeded at a density of 4.4×10^5 cells per cm^2 . The culture medium consisted of:

- 1 to 3 day: DHEM-HG + 1% (v/v) non-essential amino acids + 0.04 mg/mL of gentamycin + 20% (v/v) iFBS;
- 3 to 7 day: DHEM-HG + 1% (v/v) non-essential amino acids + 0.04 mg/mL of gentamycin + 15% (v/v) iFBS;
- 7 to 22 day: DHEM-HG + 1% (v/v) non-essential amino acids + 0.04 mg/mL of gentamycin + 10% (v/v) iFBS.

Confluent monolayers of differentiated cells were obtained after 20–22 days. The culture medium was changed twice a week. The integrity of Caco-2 monolayers was monitored twice a week by measuring transepithelial electrical resistance (TEER) using Millicell – ERS (Electrical Resistance System) (Merck Millipore, Germany).

When the confluent monolayer was obtained (after 20–22 days), the medium was removed and cells were washed three times with the warm Hanks' Balanced Salt solution containing calcium and magnesium [HBSS, pH 7.4] (Sigma Aldrich, USA), and then the cells were pre-incubated with a buffer at 37°C for 30 min. Then, TEER values were measured

for each well, and those with TEER values less than 500 ohms/ cm^2 were discarded.

After pre-incubation, the buffer was removed, and the proper solutions of genistein or its derivatives (25 μM) were added either to the apical (transport A-B), or to the basolateral (transport B-A) compartment. Stock solutions of compounds were prepared in DMSO. The concentration of DMSO (Acros Organics, USA) in transport medium did not exceed 0.01% (v/v). Fresh HBSS without analyzed compounds was added to basolateral (transport A-B) or apical (transport B-A) compartment in a well serving as a blank sample.

The samples were collected from basolateral (transport A-B) or apical (transport B-A) chambers after 1, 2, 4, 8 and 24 h incubation of cells with the tested substances. Moreover, after 24 h, samples were collected from apical (transport A-B) and basolateral (transport B-A) chamber. Each time after collecting the samples, the volume of HBSS, equal to the volume of the sample collected was added to culture solution. After 24 h, TEER value was measured to confirm the integrity of the monolayer.

Afterwards, the samples were centrifuged ($2000 \times g$, 2 min). Supernatant (100 μL) was filtered through a syringe filter (0.22 μL , 4 mm, nylon) (Thermo Scientific, USA), and then the filter membrane was washed by acetonitrile (100 μL) (Merck Millipore, Germany). The filtrates were mixed and transferred to an autosampler vial. The concentrations of tested compound were determined by HPLC-MS/MS.

The experiments evaluating the rate of transport in apical-basolateral (AP-BL) and basolateral-apical direction were performed in triplicates.

The permeability of a compound was calculated using the following equation:

$$P_{app} = \frac{dC}{dt} \cdot \frac{1}{C_0 \cdot A},$$

where dC/dt is the rate of drug transport (mol/s), A is the surface area of membrane (cm^2), C_0 is the initial concentration in the donor chambers (mol/L). Efflux ratio was calculated according to the following equation (11, 12):

$$P_{ratio} = \frac{P_{app, B-A}}{P_{app, A-B}}$$

Chromatographic conditions for determination of genistein and its derivatives

Liquid chromatography

The LC separation was performed using a Dionex UHPLC system (Dionex Corporation, USA)

consisting of an UltiMate 3000 RS pump, an UltiMate 3000 autosampler, an UltiMate 3000 column compartment and an UltiMate 3000 variable wavelength detector. UHPLC system was operated using Dionex Chromaleon™ 6.8 software.

Chromatography was performed using a C18 ACE column (150 × 4.6 mm, 3.0 μm) (Advanced Chromatography Technologies, UK) connected by the integral holder (3.2 × 4.6 mm) with the guard column of the same material. Isocratic conditions were: 30% of 0.1% solution of formic acid (Sigma Aldrich, Fluka, Germany) in water (v/v) (Merck Millipore, Germany) and 70% of acetonitrile (Merck Millipore, Germany) for genistein, Gen-X and Gen-X' or 15% of 0.1% solution of formic acid in water (v/v) and 85% of acetonitrile for G21, the flow rate for all compounds was set at 1.0 mL/min and sample injection volume was 5 μL. The HPLC column was thermostated at 25°C. Samples were kept in the autosampler at 10°C.

Mass spectrometry

For determination of compound mass, the UHPLC system was connected to a 4000 Q TRAP triple quadrupole, linear ion trap mass spectrometer (Applied Biosystem/ MDS SCIEX, USA). For data acquisition, Analyst software (version 1.5.1.) was used. The mass spectrometer was operated in the negative (for genistein) or positive (for Gen-X, Gen-X' and G21) electrospray ionization (ESI) mode.

In order to obtain the best performance of mass spectrometer analysis of genistein derivatives, the parameters dependent on the source, such as: ion source gas 1 (GS1), ion source gas 2 (GS2), curtain gas (CUR), ion spray voltage (IS) and temperature of the heater gas (TEM) were optimized. The high-pressure nitrogen was used as ion source gas, curtain gas and collision gas.

Moreover, to get the good sensitivity and the peak shape, the compound dependent parameters, such as: declustering potential (DP), entrance poten-

Table 1. MS/MS conditions for determination of genistein and its derivatives.

Analyte	t _r (min)	Q1 (m/z)	Q3 (m/z)	DP (V)	EP (V)	CE (V)	CXP (V)	CUR (ř)	IS (V)	TEM (°C)	GS1 (ψ)	GS2 (ψ)
Genistein	1.90	268.9	132.9	-95	-10	-44	-5	10	-4000	600	70	50
Gen-2	1.85	314.9	271.2	100	6	30	20	10	4500	600	70	50
Gen-3	1.96	329.0	271.2	100	7	35	16	10	4500	600	70	50
Gen-5	2.21	357.0	271.1	100	10	29	6	10	4500	600	70	50
Gen-2'	1.87	315.0	271.1	90	9	30	20	10	4500	600	70	50
Gen-3'	1.98	329.0	270.8	100	7	35	16	10	4500	600	70	50
Gen-5'	2.22	357.1	270.8	100	10	29	6	10	4500	600	70	50
G21	1.84	771.3	331.2	100	7	20	10	10	5500	550	70	50

t_r – retention time, Q1- precursor ion, Q3 – fragment ion

Table 2. MRM transitions for determination of metabolites of genistein and its derivatives.

Analyte	Q1 (m/z)	Q3(m/z)
Genistein sulfate	349	269
Genistein glucuronide	445	269
Gen-2, Gen-2' sulfates	395	315
Gen-2, Gen-2' glucuronide	491	315
Gen-3, Gen-3' sulfates	409	329
Gen-3, Gen-3' glucuronide	505	329
Gen-5, Gen-5' sulfates	437	357
Gen-5, Gen-5' glucuronide	533	357
G21 sulfate	851	271or 771
G21 glucuronide	947	271 or 771

tial (EP), collision energy (CE), and collision cell exit potential (CXP) were tuned up for individual compounds. Optimized parameters are summarized in Table 1. Final analysis was performed in a selected reaction monitoring mode, using the precursor ions and the corresponding product ions.

Chromatographic conditions for detection of metabolites

Sample preparation

To determine the possible metabolites, samples collected after 24 h of incubation were mixed and lyophilized. Residues were dissolved in 100 μ L mixture of acetonitrile : water, 4 : 1 (v/v) and filtered through a syringe filter. The filter membrane was washed with acetonitrile (100 μ L). Mixed filtrates were transferred to an autosampler vial and analyzed by HPLC-MS/MS.

Liquid chromatography

The analysis of metabolites was performed using HPLC system, software and column described

previously. The eluents were composed of 0.1% solution of formic acid in water (A) and acetonitrile (B). The elution conditions were as follows: 0 min 10% B, 0–3 min 20% B, 3–6 min 40% B, 6–10 min 50% B, 10–11 min 60% B, 11–12 min 70% B, 12–15 min 80% B for genistein, Gen-X and Gen-X' or 0 min 10% B, 0–3 min 20% B, 3–6 min 40% B, 6–8 min 50% B, 8–10 min 60% B, 10–12 min 70% B, 12–15 min 80% B for G21. The flow rate for all compounds was set at 0.8 mL/min and sample injection volume was 10 μ L. The HPLC column was thermostated at 25°C. Samples were kept in the autosampler at 10°C.

Mass spectrometry

Analyses of conjugates with glucuronic or sulfuric acid were performed on a selected reaction monitoring mode. The MRM transitions for genistein was found in the literature (13). In case of genistein derivatives, analyses were performed on theoretical MRM transitions determined based on data available for genistein (Table 2). The MS/MS con-

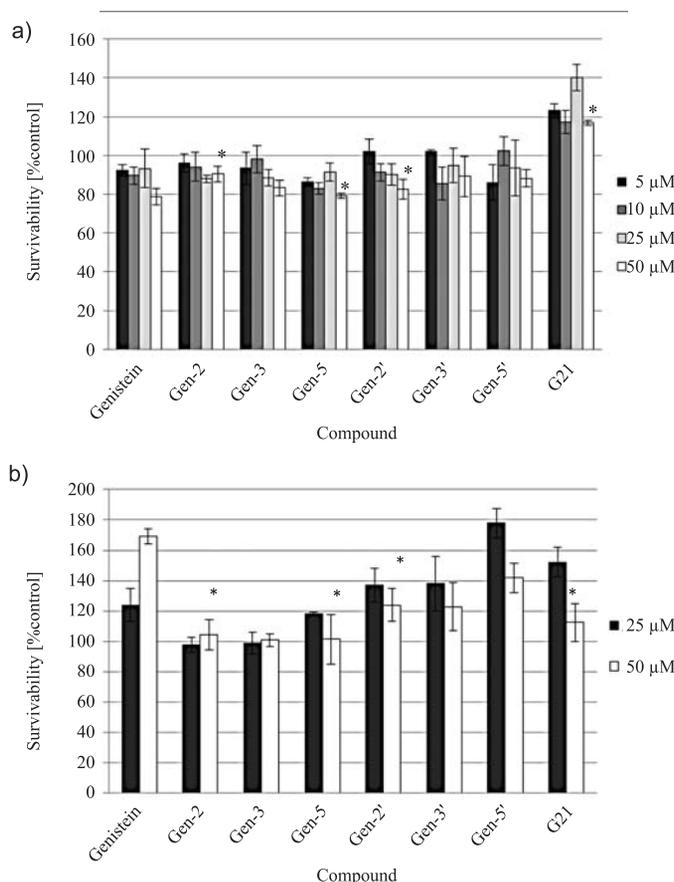


Figure 2. Percent of cell proliferation in relation with concentration of compound; a) compound solution in medium b) compound solution in HBSS; “*” compound precipitated in solution

ditions were described previously. Moreover, for identification of metabolites the LightSight software (version 2.2.1.) was used.

RESULTS AND DISCUSSION

Cells cultures are playing very important role in drug bioavailability studies. Many papers show a good correlation between transport of compounds by the intestinal epithelium *in vitro*, and by the intestinal wall *in vivo*. One of the most common cell line used in drug transport studies is Caco-2. This cell line shows many characteristics of human small intestine epithelium and serves as an *in vitro* model of drug transport through the intestine wall (14). Caco-2 cells form tight junctions on lateral side of plasma membrane, produce enzymes, taking part in transport and metabolism of compounds, and excrete small amounts of intestinal mucus on the apical surface (14, 15).

In this study, the bioavailability and metabolism were determined for six genistein derivatives, in which genistein is linked at C-7 or C-4' position with an alkyl chain containing two, three or five carbon atoms by *O*-glycosidic bond and one pyranosyl derivative of genistein (G21). The study was started with determination of cytotoxicity of the tested compounds in Caco-2 cells, which allowed us to deter-

mine the highest nontoxic concentration of the tested compounds. The highest non-toxic concentration of the drug was used in experiments aiming at evaluation of transport through cell monolayer measured with HPLC-MS/MS. The toxicity of the tested compounds in Caco-2 cell line was determined with use of MTT assay.

MTT assay (Fig. 2) showed, that the analyzed compounds used in concentration up to 50 μM (solutions prepared in culture medium) affected cell viability only by about 10%, compared to untreated control. Due to low solubility of certain compounds and their precipitation in culture medium, Gen-2, Gen-5, Gen-2' and G21 were used at 25 μM . MTT assay showed that 25 μM solutions of these drugs were safe for Caco-2 cell monolayer and it was selected for absorption and metabolism experiments. Interestingly, when cells were incubated with HBSS solution of the tested genistein derivatives, the metabolic activity exceeded the control values, suggesting that these compounds stimulated cell metabolism.

We found that all the analyzed compounds permeated across the membrane to the acceptor compartment in both the apical to basolateral and the basolateral to apical direction and they were highly permeable. P_{app} values obtained for both directions of transport were greater than $1 \times 10^{-6} \text{ cm/s}$, suggest-

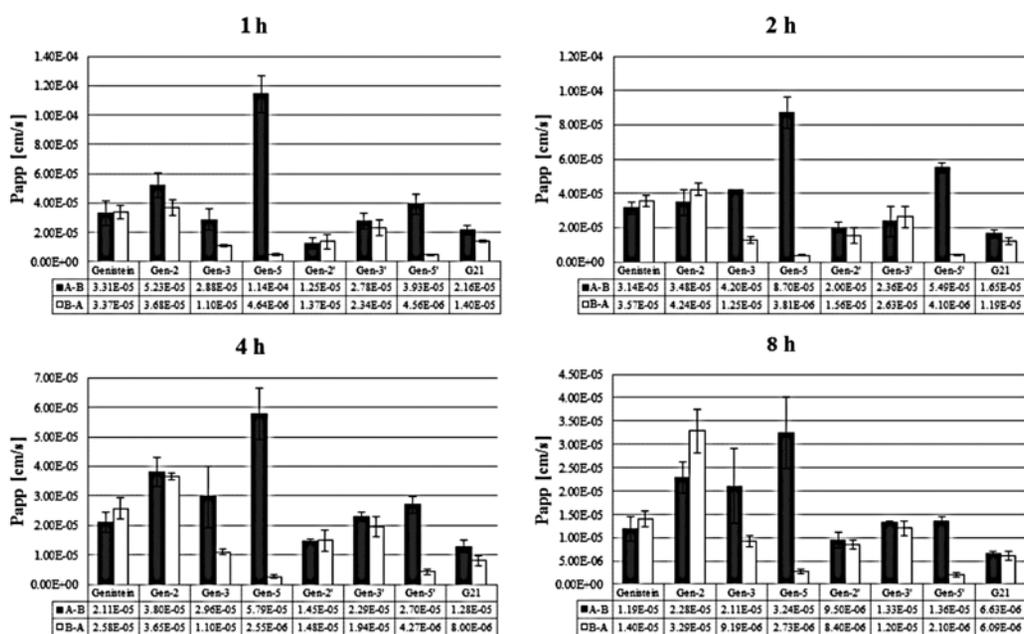


Figure 3. Apparent permeability values obtained for AP-BL and BL-AP directions in the Caco-2 after 1, 2, 4 and 8 h of incubation

Table 3. Retention times for glucuronide and sulfate conjugates of genistein and its derivatives.

	Retention time [min]		
	Parent	Glucuronide	Sulfate
Genistein	12.51	8.98	11.28
Gen-2	11.91	9.0	10.49
Gen-3	13.09	9.61	11.37
Gen-5	14.61	11.39	12.94
Gen-2'	12.20	8.89	10.90
Gen-3'	13.31	9.48	11.33
Gen-5'	14.69	10.49	13.63
G21	14.17	–	12.75

Table 4. Values of efflux ratio for genistein and derivatives after 1, 2, 4 and 8 h of incubation.

	Efflux ratio			
	1 h	2 h	4 h	8 h
Genistein	1.02	1.13	1.22	1.18
Gen-2	0.70	1.22	0.96	1.44
Gen-3	0.38	0.30	0.37	0.44
Gen-5	0.04	0.04	0.04	0.08
Gen-2'	1.10	0.78	1.02	0.88
Gen-3'	0.84	1.12	0.85	0.90
Gen-5'	0.12	0.07	0.16	0.15
G21	0.65	0.72	0.63	0.92

ing that these compounds will be completely absorbed in human after administration per os. According to the literature (16), absorption of drugs in human is correlated with permeability coefficient in the following way:

- P_{app} : $> 1.0 \times 10^{-6}$ cm/s – drugs are absorbed in 100%
- P_{app} : $0.1-1.0 \times 10^{-6}$ cm/s – drugs are absorbed to a greater than 1% but less than 100%
- P_{app} coefficient: 1.0×10^{-7} cm/s – drugs are absorbed less than in 1%.

The values of P_{app} obtained for genistein were similar to values found in the literature (17, 18). Calculated $P_{app, A-B}$ values for most of genistein derivatives (except for Gen-2' and G21) were higher in comparison with P_{app} value for genistein. For derivatives of Gen-X and Gen-X' series, the value of coefficient increased with increasing number of carbon atoms in a linker (except for Gen-3). The highest value of $P_{app, A-B}$ was obtained for Gen-5. Of note, this compound showed the best anticancer proper-

ties (the lowest IC_{50} value) (7, 8), among both, Gen-X and Gen-X' series.

Apparent permeabilities in the mucosal direction ($P_{app, B-A}$) were for most derivatives lower than the values obtained for genistein. They were also lower than the $P_{app, A-B}$ values obtained for the transport in the opposite direction. It is also worth to note that derivatives of Gen-X series (substituted at C-7 position of genistein) have higher values of $P_{app, A-B}$ and $P_{app, B-A}$ coefficients when compared to their analogs of Gen-X' series (substituted at C-4' of genistein).

The results presented in Figure 3 show clearly that P_{app} values changed depending on duration of incubation with drugs. This observation can be explained by the metabolic changes of the analyzed compounds that occurred in Caco-2 cells. On the base of literature data, Caco-2 cells are known to secrete enzymes capable to metabolize drugs by connecting to their structure glucuronic acid or sulfuric acid (14).

Following this assumption, we analyzed the samples collected 24 h after addition of a drug, and we found that genistein and its derivatives were metabolized, and the type of derivatization was dependent on the type of molecule. Genistein and its derivatives containing the alkyl chain were metabolized by conjugation of sulfuric acid or glucuronic acid. (Table 3). The only metabolite of G21 detected in our analysis was its sulfate. The obtained results are only of qualitative character; quantitative analysis of the metabolites formed in Caco-2 cells was not possible due to the lack of appropriate standards.

Determination of $P_{app, A-B}$ and $P_{app, B-A}$ coefficient for the tested drugs allowed us to determine the P_{ratio} values, helpful in predicting drug transport back into the intestinal lumen. It is assumed that a P_{ratio} greater than 2 is predictive of relevant efflux (active drug transport back into the intestinal lumen) (11, 19). The P_{ratio} of genistein derivatives (Table 4) were lower than for genistein and lower than 2. The smallest values of these coefficient were obtained for Gen-5 and Gen-5' (after 1 h of incubation: 0.04 and 0.12, respectively). It means that this drug may become a useful leader in the future drug development.

In conclusion, modifications introduced to genistein structure influenced not only antitumor activity of compounds (lower IC_{50} values in comparison to genistein), but also improved their availability parameters (increased permeability coefficient and decreased P_{ratio} values).

In the perspective, we plan to study permeability and metabolism of new analogs of Gen-X and Gen-X', which contain a sugar moiety – rhamnol, connected with an isoflavone by a glycosidic bond. These derivatives showed higher antitumor activity in comparison to compounds of Gen-X or Gen-X' series. Since the sugar moiety may influence the biological properties of a compound remarkably, it can be assumed that this type of derivatization will alter transport and bioavailability of genistein derivatives.

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