Research Article

Genistein Derivatives Regioisomerically Substituted at 7-O- and 4'-O- Have Different Effect on the Cell Cycle

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Our previous studies on antiproliferative properties of genistein derivatives substituted at C7 hydroxyl group of the ring A revealed some compounds with antimitotic properties. The aim of this work was to synthesize their analogues substituted at the 4'-position of the ring B in genistein and to define their antiproliferative mechanism of action in selected cancer cell lines *in vitro*. C4'-substituted glycoconjugates were obtained in a three-step procedure: (1) alkylation with an ω -bromoester; (2) deacylation; (3) Ferrier-type rearrangement glycosylation with acylated glycals. Biological effects including antiproliferative effects of the compounds, cell cycle, DNA lesions (ATM activation, H2A.X phosphorylation, and micronuclei formation), and autophagy were studied in human cancer cell lines. Some of the tested derivatives potently inhibited cell proliferation. The presence of a substituent at the 4'-position of the ring B in genistein correlated to a p53-independent G1 cell-cycle arrest. The derivatives substituted at C4' did not induce DNA lesions and appeared to be nongenotoxic. The tested compounds induced autophagy and caused remarkable decrease of cell volume.

1. Introduction

Flavonoids are naturally occurring plant polyphenols found in abundance in fruits, vegetables, and plant-derived beverages [1]. Epidemiological studies have shown that the intake of certain vegetables, fruits, and tea in the daily diet provides effective cancer prevention. This class of compounds have been found to arrest cell-cycle progression either at G1/S or at G2/M boundaries [2] and inhibit cell proliferation. The inhibition of cell-cycle is a result of inhibition of different cell-cycle kinases or activation of cell-cycle control checkpoints [3–5].

Beneficial influence of genistein on human health, resulting from its interaction with multiple molecular targets [6, 7] combined with opportunities of chemical functionalization, makes this molecule very attractive as a lead compound. The structure of genistein molecule enables different types of

derivatizations, that is, modification of the isoflavone skeleton or manipulation functional groups around the isoflavone core, which may improve its affinity towards molecular targets [8].

There are many examples of genistein derivatives which show better pharmacological characteristics comparing to the parent compound genistein, including altered binding affinities to estrogen receptors (ERs) [9] and enhanced antiproliferative activities [10–14]. Among genistein derivatives showing enhanced antiproliferative activity are glycoconjugates substituted with mono- and disaccharides at the position C7 of the ring A of genistein [7, 10, 12]. Most of these compounds inhibited cell proliferation by the cell-cycle arrest in G2 phase [11, 15]; however, some of them exhibited unique properties for isoflavonoids and affected microtubule assembly at low micromolar concentration [10, 11, 16]. In

contrast, genistein did not affect microtubules at all [16–18] or show weak microtubule depolymerizing effects at high micromolar concentration [19]. Both, the type of a sugar moiety and a linker between isoflavonoid and its substituent were important for this unique activity of derivatives against microtubules [11, 17].

Hitherto, most of the described derivatives were obtained by a substitution of a C7 hydroxyl group hydrogen atom. The synthesis of C4'-substituted compounds was not studied frequently and biological activity of these compounds is poorly understood. It has been demonstrated [20] that selective functionalization of 4'-hydroxyl group in genistein requires initial double deprotonation with a strong base because resulting dianion exhibits different nucleophilicity at both phenolate centers, favoring preferred direction of O-alkylation. Potassium *tert*-butanolate is believed to be the best base for this task, but genistein has limited stability under strong alkaline conditions and butanolate anion can react with an alkylating reagent; both factors diminish effectiveness of such reaction. In our previous paper [21], we achieved a scalable, selective alkylation of genistein, enabling secondary functionalization needed for preparation of more extended structures.

In this paper, we presented the synthesis of genistein glycoconjugates substituted at the 4'-position of the ring B and described the biological effects of these compound on cancer cell lines. We showed that antiproliferative effects of C4' substituted derivatives of genistein are related to the mechanism different than that described for C7 substituted regioisomers [11]. Antiproliferative effects of C4' derivatives are mediated at least in part by arrest of cell-cycle progression at G₁-S. The cell-cycle arrest is not related to DNA damage checkpoint activation and the tested derivatives are not genotoxic.

2. Material and Methods

2.1. Synthesis of Genistein Derivatives

2.1.1. General Methods. ¹H and ¹³C NMR spectra were recorded (DMSO-d₆ solution) with TMS internal standard on 600 MHz Varian Inova 600 MHz apparatus with Me₄Si as internal reference. Optical rotations were measured with a Perkin-Elmer 141 polarimeter using a sodium lamp (589 nm) at room temperature. Mass spectra (HRMS) were recorded in the positive mode on a Mariner (PerSeptive Biosystems) detector using the electrospray-ionization (ESI) technique. Reactions were monitored by TLC on precoated plates of silica gel G (Merck); components were detected under UV light or by charring with 10% sulphuric acid in ethanol. Column chromatography was performed on silica gel 60 (70–230 mesh, E. Merck) developed with one of the toluene/ethyl acetate or hexane/ethyl acetate solvent systems. All evaporations were performed under diminished pressure at 50°C.

2.1.2. Chemicals. The reagents (chemicals), all being of A.R. grade, were purchased from Merck Company, 5,7-dihydroxy-3-(4-hydroxyphenyl)-4H-chromen-4-one, Genistein (1,

anhydrous, >99%) provided by Pharmaceutical Research Institute (Warsaw, Poland), was refined prior to use by its recrystallization from ethanol (pale yellow needles, m.p.: 298–299°C).

¹H NMR (DMSO-d₆). 6.21 (d, J 1.9 Hz, 1H), 6.37 (d, J 1.9 Hz, 1H), 6.80 (d, J 1.9 Hz, 2H), 7.35 (d, J 8.5 Hz, 2H), 8.32 (s, 1H), 9.64 (s, 1H), 10.94 (s, 1H), 12.96 (s, 1H). HRMS [M + Na]⁺ 300.0530, Anal. Calcd for C₁₅H₁₀O₅: C, 66.67; H, 3.73. Found: C, 66.68; H, 3.87.

2.1.3. Regioselective Alkylation of Genistein. Hydroxyalkyl derivatives of genistein were prepared according to procedures described elsewhere (Figure 1) [21]. Obtained products were characterized by the following data:

5,7-dihydroxy-3-(4-(2-hydroxyethoxy)phenyl) chromen-4H-one (**4a**; Gen-2'), white solid, m.p.: 210°C, decomposition; HRMS [M + Na]⁺ 337,0643

5,7-dihydroxy-3-(4-(3-hydroxypropoxy)phenyl) chromen-4H-one (**4b**; Gen-3'), white solid, m.p.: 206–208°C, decomposition; HRMS [M + Na]⁺ 351,0655

5,7-dihydroxy-3-(4-(5-hydroxypentyloxy)phenyl) chromen-4H-one (**4c**; Gen-5'), white solid, m.p.: 200–202°C, decomposition; HRMS [M + Na]⁺ 379,0949.

2.1.4. Synthesis of Glycoconjugates

(1) General Procedure. To a solution of glycosyl acceptor, 4(4'-hydroxyalkyloxy) genistein; 1.5 mMol) and 3,4-di-O-acetyl-L-rhamnal, **5** (0.43 g, 2 mMol) in anhydrous acetonitrile (20 mL) catalyst InCl₃ (4.4 mg, 0.02 mMol) was added, and mixture was stirred at room temperature. Reaction was monitored by TLC (chloroform/methyl alcohol = 10/1, v/v). Reaction mixture was filtered, washed with methylene chloride, aqueous solution of NaHCO₃, and water until becoming neutral. Organic solution was dried (MgSO₄), filtered, and concentrated to give crude product. Crude product was purified by column chromatography. (chloroform/methyl alcohol = 200/1) to yield **6a**, **6b**, and **6c** (Figure 2).

(2) 5,7-Dihydroxy-3-[4-(4-O-acetyl-2,3,6-trideoxy-α-L-erythro-heks-2-enopyranosyl)-2-ethoxyphenyl]-chromen-4-one, (6a**; Ram-2').** Product, colorless syrup (450 mg, yield 64%).

HRMS (ESI): calculated C₂₅H₂₄O₉ [M + Na]⁺ 491.1318, determined 491.1324.

¹H NMR δ (CDCl₃). 1.24 (d; 3H; J = 6.6 Hz; H-6''), 2.10 (s; 3H; AcO), 3.91–3.95 (m; 1H; H-5''), 4.04 (dq; 1H; J = 9.2 Hz, J = 6.2 Hz; –CH₂O–), 4.08–4.22 (m; 3H; 2x –CH₂O–), 5.07, 5.09 (2x m; 2H; H-1''β, H-4''), 5.85, 5.87 (2x m; 2H; H-2'', H-3''), 6.25, 6.30 (2x d; 2H; J = 2.2 Hz; H-6, H-8), 6.94 (AA'XX';

FIGURE 1: Synthesis of 4'-(hydroxyalkyl) genistein.

FIGURE 2: Synthesis of glycoconjugates derivatives of genistein.

2H; J = 9.0 Hz; H-3', H-5'), 7.40 (AA'XX'; 2H; J = 9.0 Hz; H-2', H-6'), 7.65 (br s; 1H; 7-OH), 7.81 (s; 1H; H-2), 12.85 (s; 1H; 5-OH).

(3) 5,7-Dihydroxy-3-[4-(4-O-acetyl-2,3,6-trideoxy-α-L-erythro-heks-2-enopyranosyl)-3-propyloxyphenyl]-chromen-4-on, (**6b**; Ram-3'). Product, colorless syrup (580 mg, yield 80%).

HRMS (ESI): calculated C₂₆H₂₆O₉ [M + Na]⁺ 505.1475, determined 505.2004.

¹H NMR δ (CDCl₃). 1.21 (d; 3H; J = 6.3 Hz; H-6''), 2.09 (m; 2H; -CH₂-), 2.09 (s; 3H; AcO), 3.70 (dt; 1H; J = 9.9 Hz, J = 6.1 Hz; -CH₂O-), 3.94-3.99 (m; 2H; H-5'', -CH₂O-), 4.09 (t; 2H; J = 6.2 Hz; -CH₂O-), 4.99 (br s; 1H; H-1''α), 5.06 (m;

1H; H-4''), 5.86 (br d; 1H; J = 10.3 Hz; H-2''), 5.81 (ddd; 1H; J = 10.3 Hz, J = 4.5 Hz, J = 2.2 Hz; H-3''), 6.27, 6.33 (2x d; 2H; J = 2.1 Hz; H-6, H-8), 6.78 (br s; 1H; 7-OH), 6.95 (AA'XX'; 2H, J = 8.7 Hz; H-3', H-5'), 7.42 (AA'XX'; 2H; J = 8.7 Hz; H-2', H-6'), 7.82 (s; 1H; H-2), 12.89 (s; 1H; 5-OH).

(4) 5,7-dihydroxy-3-[4-(4-O-acetyl-2,3,6-trideoxy-α-L-erythro-heks-2-enopyranosyl)-5-pentyloxyphenyl]-chromen-4-on, (**6c**; Ram-5'). Product, colorless syrup (665 mg, yield 87%).

HRMS (ESI) calculated C₂₈H₃₀O₉ [M + Na]⁺ 533.1788, determined 533.2104.

¹H NMR δ (CDCl₃). 1.24 (d; 3H; J = 6.0 Hz; H-6''), 1.51-1.60 (m; 2H; -CH₂-), 1.64-1.74 (m; 2H; -CH₂-), 1.76-1.86 (m; 2H;

FIGURE 3: Inhibition of proliferation of cancer cells by genistein derivatives substituted at C4'.

FIGURE 4: Cell-cycle phases distribution in HCT 116 and DU 145 cells treated with genistein derivatives substituted at C4'.

–CH₂–), 2.10 (s; 3H; AcO), 3.54 (dt; 1H; J = 9.7 Hz; J = 6.4 Hz; –CH₂O–), 3.82 (dt; 1H; J = 9.7 Hz; J = 6.6 Hz; –CH₂O–), 3.97 (t; 2H; J = 6.5 Hz; –CH₂O–), 3.93–4.02 (m; 1H; H-5''), 4.97–5.00 (m; 1H; H-1''α), 5.07 (ddd; 1H; J = 9.2 Hz; H-4''), 5.82, 5.86 (2x m; 2H; H-2'', H-3''), 6.23, 6.33 (2x d; 2H; J = 2.1 Hz; H-6, H-8), 6.93 (AA'XX'; 2H; J = 9.0 Hz; H-3', H-5'), 7.36 (br s; 1H; 7-OH), 7.40 (AA'XX'; 2H; J = 9.0 Hz; H-2', H-6'), 7.82 (s; 1H; H-2), 12.87 (s; 1H; 5-OH).

2.2. Cell Lines, Culture Conditions, and Treatment

2.2.1. Cell Lines. Human prostate cancer cells DU 145 and human colon cancer cells HCT 116 wt were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). HCT 116 –/– p53 (both p53 alleles deleted by targeted homologous recombination) [22] were a gift from Dr. Bert Vogelstein. PC3 cells stably expressing EGFP-LC3 were a

FIGURE 5: Cell-cycle phases distribution in HCT 116 -/- p53 cells treated with genistein derivatives substituted at C4'.

gift from Dr. Anna Herman-Antosiewicz. Cells were grown in RPMI 1640 medium (Sigma-Aldrich, Germany) supplemented with 10% (v/v) inactivated fetal bovine serum (FBS) (GIBCO Invitrogen, UK) and 40 µg/mL gentamicin sulfate (Sigma-Aldrich, Germany) at 37 °C in humidified atmosphere with 5% CO₂. In order to achieve the desired concentrations of the tested compounds, the stock solutions were mixed with fresh medium. Final DMSO concentration in culture media was less than 0.2%. Cells grown in nonmodified medium with 0.2% DMSO served as a control. This concentration of DMSO did not alter cell growth rate and cell-cycle, as compared with the vehicle-free medium.

2.2.2. MTT Assay. The assay was performed as described previously [11].

2.2.3. Flow Cytometry Analysis of Cell-Cycle. Cycle phases distribution after 24 h treatment with genistein derivatives was performed as described previously in [11].

2.2.4. Western Blotting. p53, phospho-p53 (Ser15) and p21, phospho-ATM (Ser 1981) were detected as described previously in [23].

2.2.5. Immunofluorescent Detection of Histone γH2A.X. Immunocytochemistry was performed as described previously in [16]. Photographs were taken under NIKON Eclipse E800 fluorescent microscope equipped with HAMAMATSU C5810 camera. Analysis of mean fluorescence was performed with a CometScore software.

2.2.6. Comet Assay. Cells were plated in culture dishes Ø 30 mm (NUNC) and treated with the tested compounds for 24 h. Adherent cells were harvested by trypsinization. 2 × 10⁵ cells were suspended in 50 µL medium. Low melting agarose (BioAmerica) was added to the cell suspension (final

concentration of agarose was 1%); 100 µL of the suspension was dropped to the microscope slide covered with 0.5% normal melting point agarose (Lonza). The slide was left on ice for 15 minutes. Alkaline assay, enabling single-strand breaks (SSB) detection, was performed according to the procedure described elsewhere [24]. In brief, slides were immersed in lysing solution (5 M NaCl, 0.5 M EDTA, 10 mM Trizma base, 100% Triton X-100, pH > 10) for 1 h at 4 °C; the electrophoresis was run for 15 min. in 4 °C at 0.8–1.0 V/cm and 300 mA; then the slides were neutralized with a neutralizing buffer (1 M Trizma base, pH 7.5) for 5 minutes. This step was repeated twice. Neutral assay for double-strand breaks (DSB) detection was performed according to the procedure described elsewhere [25] with minor modifications. Microscope slides were immersed in lysing solution (2.5 M NaCl, 0.1 M EDTA, 10 mM Trizma base, 0.5 Triton X-100, 1% Tween 20, 10% DMSO, pH 9.5) for 2 h at 4 °C and neutralized (0.5 M H₃BO₃, 0.5 M EDTA, 1 M Trizma base, pH 8.5) for 1 h at 4 °C. The electrophoresis was run at 4 °C for 25 minutes at 12 V, 7 mA. Slides were drained with cold 96% ethanol for 10 minutes at 4 °C and stained with ethidium bromide (2 µg/mL). The slides were analyzed with a fluorescent microscope; images were recorded with a Hamamatsu C5810 camera and analyzed with a CometScore software.

2.2.7. Micronucleus Assay. Cells were grown on coverslips in 3 cm plates for 24 h. Then the medium was replaced with the one containing cytochalasin B (2 µg/mL) and genistein or its derivatives, and cells were incubated for 24 hours in the cell culture incubator. After washing with cold, PBS cells were fixed in ice-cold methanol (–20 °C) for 10 minutes, air-dried, washed with PBS, and stained with 1 µg/mL 4'-6'-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma-Aldrich, Germany). Slides were mounted in DAKO Fluorescent Mounting Medium (Dako, USA). To score micronuclei (MN), 1000 DAPI-stained binucleated cells were examined in each well by fluorescence microscopy using the ×40 objective. According to Fenech [26], micronuclei were scored when their diameter was less than one-third of the mean diameter of the nucleus.

2.2.8. Analysis of Intracellular Pattern of EGFP-LC3. PC3 cells were seeded in 8-well chamber slides (Nunc, NY, USA) and left for 24 h. Then the medium was aspirated from the chamber slide wells and replaced with warm medium with the tested compounds; cells were then incubated for 24 h. Z-stacks were acquired with the Zeiss LSM 710 inverted confocal microscope with the 488 nm excitation laser and Zeiss Plan-Apo oil immersion 63x objective. Consecutive optical sections were superimposed with Zen software in order to show vesicular pattern across the whole cell depth.

3. Results

3.1. Synthesis of Genistein Glycoconjugates. Genistein, like other flavonoid polyphenolic compounds, is a notoriously

FIGURE 6: Index of binucleated cells representing completed nuclear divisions counted after 24 h treatment with genistein or its C4'-substituted derivatives used at concentrations corresponding to 2x IC₅₀ and 3x IC₅₀.

difficult substrate for chemical glycosylation, including Ferrier rearrangement of glycals, and regio- as well as stereoselectivity is practically unattainable [12]. As a consequence, "through-spacer" derivatization methodology has

been developed by using bifunctional aliphatic linkers of various lengths [8]. The main principle of such derivatization, as exemplified by reactions of **1** with ω -benzyloxyalkylbromides exploited in this paper, is consistent with the observation

FIGURE 7: ATM activation in HCT 116 wt cells treated with genistein (100 μ M), camptothecin CPT (1 μ M), and genistein derivatives substituted at C4' at the concentration equivalent to 3x IC_{50} (Ram-2' (3 μ M), Ram-3' (40 μ M), Ram-5' (20 μ M), Gen-5' (20 μ M). HSC 70 served as a loading control.

FIGURE 8: The level of total p53, phosphorylated p53 (ser 15), p21 and MDM2 in HCT 116 wt cells treated for 24 h with the tested genistein derivatives used at the concentration equivalent to 3x IC_{50} (Ram-2' (3 μ M), Ram-3' (40 μ M), Ram-5' (20 μ M), Gen-5' (20 μ M). Genistein (Genist) and camptothecin (CPT) were used as positive controls. HSC 70 served as a loading control.

by Lewis et al. [20] that 7-O: 4'-O-dianions exhibit reverse selectivity for alkylation of those 7-O monoanions. Consequently, a parallel series of selectively substituted monoalkyl 7-O and 4'-O-isoflavones could be secured for biological activity studies [21].

3.2. Antiproliferative Activity of Derivatives. In the first step we evaluated the influence of derivatives of genistein substituted at C4' on the proliferation of HCT 116 and DU 145 cells after 72 h treatment with MTT assay (Figure 3).

On the basis of experimental data, we calculated the value of IC_{50} (proliferation inhibited to 50% of control) using CalcuSyn Software based on minimal square fitting to 4-parameter logistic curves. Mean IC_{50} value and standard deviation were then calculated from 3–5 independent experiments (Table 1). We found four compounds: Gen-5', Ram-2', Ram-3', and Ram-5' showing the activity significantly higher than genistein.

TABLE 1: IC_{50} value of proliferation inhibition calculated on the base of MTT assay.

	IC_{50} (μ M)	
	HCT 116 wt	DU 145
Gen-2'	>100	>100
Gen-3'	25.58 $\pm$ 10.81	21.60 $\pm$ 10.92
Gen-5'	7.19 $\pm$ 2.04	6.42 $\pm$ 2.87
Ram-2'	0.07 $\pm$ 0.03	0.37 $\pm$ 0.10
Ram-3'	13.61 $\pm$ 2.20	12.59 $\pm$ 5.43
Ram-5'	7.17 $\pm$ 1.76	6.04 $\pm$ 1.25

3.3. The C4'-Substituted Derivatives Arrest a Cell-cycle at G1 Phase. In the next step we investigated the influence of the most potent derivatives on the cell-cycle after 24 h treatment. The DNA content was analyzed with the flow cytometer. The results shown in Figure 4 demonstrate that the tested compounds used at the concentration equivalent to IC_{50} and 2 $\times$ IC_{50} caused inhibition of a cell-cycle at G1 phase. The increased number of cells arrested in G1 was accompanied by the decreased number of cells in S and G2/M phases of a cycle. Very similar activity of the tested derivatives on the cell-cycle was observed in DU 145 cells (Figure 4).

DU 145 cells harbor mutations in two alleles of *p53* gene [27], so one could conceive that the observed cell-cycle effects were not dependent on this protein. In order to get deeper insight into understanding the *p53* role in G1 block induced by the investigated genistein derivatives we compared the cell-cycle in HCT 116 $-/-$ *p53* cell line with deleted *p53* and related the results to results obtained for wild *p53* HCT 116. The tested C4' derivatives, also arrested the cell-cycle in G1 phase in HCT 116 $-/-$ *p53*; however, the percentage of cells blocked in that phase was lower than in HCT 116 wt cells (Figure 5).

3.4. C4'-Substituted Derivatives Do Not Induce DNA Damage. Stress factors, among them genotoxic stress, block the cell-cycle in both *p53*-wild-type and *p53*-deficient cells [28]. Since genistein was reported to inhibit topoisomerase II, and potentially induce double-strand breaks in a similar manner to that of etoposide [29], we decided to evaluate the genotoxic activity of novel genistein derivatives, including the presence of the DNA breaks, formation of micronuclei, and activation of ATM kinase, measured as its phosphorylation on serine 1981.

We did not observe either single- or double-strand breaks measured as a moment of a tail moment in a comet assay performed in HCT 116 treated with tested derivatives (Table 2). In positive controls (genistein, etoposide, and camptothecin) there was remarkable increase of tail moment value. The immunofluorescent analysis of histone γ H2A.X showed the increase of mean fluorescence of nuclei only after treatment with etoposide (positive control) and genistein (Table 3). None of the genistein derivatives elevated the fluorescence above the control level when used at the concentration up to 3 $\times$ IC_{50} .

FIGURE 9: Autophagy in PC3 prostate cancer cell line stably expressing EGFP-LC3 (green fluorescence). Series of optical sections (Z-stack) were combined together. (a) control; (b) genistein (100 μM); (c) Gen-5' (20 μM); (d) Ram-2' (3 μM), (e) Ram-3' (40 μM), (f) Ram-5' (20 μM).

FIGURE 10: Cell-cycle arrest by differently substituted genistein derivatives.

Next, we assessed the genotoxic effects evoked by genistein derivatives with the use of micronucleus assay. The assay was performed routinely with cytochalasin B, the mycotoxin inhibiting the division of a cytoplasm, without

TABLE 2: Single-strand breaks (SSB) and double-strand breaks (DSB) detected with comet assays in HCT 116 cells.

Compound (μM)	Alkaline lysis—SSB	Neutral lysis—DSB
	Tail moment (%)	
Control (0)	6.27 ± 2.6	5.52 ± 3.18
Gen-5' (20)	3.9 ± 1.61	7.17 ± 4.82
Ram-2' (3)	5.55 ± 3.89	6.77 ± 2.22
Ram-3' (40)	5.39 ± 0.14	6.43 ± 4.29
Ram-5' (20)	3.8 ± 3.8	7.44 ± 4.17
Etoposide (50)	10.8 ± 1.82	12.63 ± 1.31
Genistein [100]	31.67 ± 3.71	0.45 ± 0.2
Camptothecin (1)	50.94 ± 5.12	1.07 ± 0.1

influencing the nuclear division, by blocking the formation of actin microfilaments necessary for cytokinesis [26]. The cells which completed the nuclear division during the treatment become binucleated and can be easily identified. This approach enables to distinguish the cells which acquired micronuclei during the treatment, from those, which already had possessed micronuclei, and is useful for assessment of

TABLE 3: γ H2A.X staining in HCT116 cells.

Treatment (μ M)	Fold change of γ H2A.X signal intensity related to untreated control
Etoposide (50)	2.45
Genistein (100)	1.4
Gen-5' (20)	0.92
Ram-2' (3)	0.70
Ram-3' (40)	0.66
Ram-5' (20)	0.91

TABLE 4: The percentage number of autophagic cells in PC3 cell line.

Treatment (μ M)	Autophagic cells (%)
Control (0)	18.5
Genistein (100)	33.2
Gen-5' (20)	64.0
Ram-2' (3)	84.6
Ram-3' (40)	94.1
Ram-5' (20)	55.5

the genotoxic properties of compounds which affect the cell-cycle. This assay demonstrated that the number of cells which completed the nuclear division during the first 24 h treatment with the tested drugs (binucleated cells) was markedly lowered in all the tested lines (Figure 6). In the micronucleus assay performed in HCT 116 wt, HCT 116 $-/-$ p53, and DU 145 cell lines, no significant increase of micronucleation was observed after treatment with genistein derivatives (Ram-2', Ram-3', Ram-5', and Gen-5') in comparison with control. In contrast, the treatment of cells with genistein caused the elevation of the number of micronucleated cells.

Next, we compared the level of ATM phosphorylation on serine 1981 in HCT 116 cells after treatment with genistein, camptothecin (CPT) and C4'-substituted derivatives (Figure 7). In contrast, to genistein and camptothecin, the C4' substituted genistein derivatives did not induce ATM kinase phosphorylation, indicating that no DNA double-strand breaks were formed (neither directly nor indirectly).

The activation of ATM by genistein and camptothecin (Figure 7) correlated with activation of p53 in HCT 116 wt cells (Figure 8). Highly elevated level of phosphorylated form of p53 (ser15) observed in cells treated with genistein was comparable with the level observed after camptothecin (CPT), a well-known activator of p53. Two of four C4' substituted genistein derivatives, Ram-2' and Gen-5', induced only slight phosphorylation of p53 at serine 15. The level of the total p53 moderately increased in cells treated with the tested compounds in comparison to the untreated control (Figure 8).

The pattern of p53 phosphorylation corresponded to p21 induction after treatment with the tested compounds (Figure 8). Genistein and camptothecin induced p21 most profoundly, Ram-2' and Gen-5' caused slight increase of that protein, while Ram-3' and Ram-5' almost did not alter p21 level, when compared to untreated control.

In parallel, we tested the level of MDM2, the cellular regulator of p53 level. Genistein did not influence the level of MDM-2 in relation to control. We observed depletion of MDM-2 by camptothecin, reported previously in [30]. Genistein derivatives, especially Ram-2' and Gen-5' elevated the level of MDM-2 in HCT 116 wt cells.

Altogether, the pattern of p53, p21, and MDM-2 expression after treatment with genistein derivatives bears striking resemblance to the pattern characteristic for AICAR, the pharmacological activator of AMP kinase [23], which is a cellular energy sensor [31]. Next, we decided to study whether the tested genistein derivatives induce metabolic stress.

3.5. C4'-Substituted Derivatives Induce Autophagy. The restriction of nutrients is a stress signal that affects cell-cycle decisions, causing the cell to arrest in G1. Usually, cells subjected to a nutrient stress induce autophagy, a process of degradation of its own organelles within lysosomes to reconstitute cellular components and supply the cell with energy [32].

We studied the influence of C4'-substituted derivatives on the process of autophagy induction using PC3 prostate cancer cell line stably expressing EGFP-LC3 protein. During autophagy, the cytoplasmic form of LC3 is processed and recruited to the autophagosomes. The hallmark of autophagic activation is the formation of cellular autophagosome puncta decorated by LC3 [33].

In control cells we observed typical, evenly distributed fluorescence of EGFP-LC3. In cells treated with genistein and genistein derivatives the induction of autophagy, visible as vesicular LC3 pattern, was detected 24 h after addition of a tested substance. The intensity of a process, expressed as the number of cells with autophagic morphology was much higher in the presence of C4'-substituted genistein derivatives than genistein (Figure 9, Table 4).

4. Discussion

The vast literature implicating antitumor activity of genistein, including induction of cancer cell differentiation, stimulation of apoptosis, inhibition of angiogenesis, and inhibition of tumor cell proliferation points tyrosine kinases and topoisomerase II as primary molecular targets of this compound [6]. Protein tyrosine kinases as targets of genistein derivatives were not within the scope of the presented work, but those interactions undoubtedly warrant intensive investigation. Comprehensive study on structure-activity relationship of a flavonoids library comprising more than 100 compounds with different substituents clearly indicates that this class represent a promising source of potent tyrosine kinase inhibitors [34, 35].

In this paper, we focused on the influence of C4'-substituted genistein derivatives on a cell-cycle and compared the results with the effects obtained previously for C7-substituted derivatives [17]. The results indicate that the regioisomeric substitutions are crucial components influencing the mode of action of genistein derivatives.

The type of cell-cycle arrest by flavonoids dependent on the structural groups was critically reviewed by Casagrande and Darbon [2]. The presence of a hydroxyl group at the C3'-position of the ring B (quercetin and luteolin) was indicated as crucial for G1 cell-cycle arrest, while its absence (kaempferol and apigenin) correlates to a G2 block. Another compound, the ester of epigallocatechin and gallic acid, (-)-epigallocatechin-3-gallate (EGCG), was also reported to block cell-cycle progression in the G1 phase [36]. Probably other structural features are also relevant to the cell-cycle arrest at G1 phase, as shown in the example of tangeretin, which stops the cycle at G1, despite the lack of the OH group at C3' [37]. In isoflavones the presence of a hydroxyl at 5-position of the ring A was suggested to play a pivotal role in the type of cell-cycle arrest. Daidzein lacking OH group at C5 induced an accumulation of cells in G1, whereas genistein arrested cells in G2. Phenoxodiol, a novel synthetic isoflavone lacking OH at C5, was also reported to block cell-cycle at G1 phase [38].

Our results indicate that regioisomeric substitutions at C4' and C7 are key regulators of the mode of isoflavonoid action. Here we show that genistein derivatives substituted with a bulky group at C4' block a cell-cycle at G1 phase, despite the presence of a hydroxyl at C5. Our previous work showed that the substitution of genistein at C7 (with unmodified hydroxyls present at C5 and C4') causes cell-cycle arrest in G2 phase [11, 15] or in mitosis [11, 17] (Figure 10).

The cell-cycle block in G2 phase caused by genistein, followed by the process of apoptosis, is related to activation of DNA damage checkpoint pathways [5]. Although epidemiological studies did not show correlation between the diet containing high amount of genistein and genotoxic effects in patients, the inhibitory activity of genistein againsts topoisomerase II is sometimes regarded as a contraindication for a broad use of this isoflavonoid in chemoprevention [39]. Genistein was shown to act as a topoisomerase II inhibitor and to cause DNA damage manifested by micronuclei and histone H2A.X staining, in a consequence of a formation of cleavable complexes [16]. The substitution of genistein with a bulky sugar group at C7 did not cancel the ability of a molecule to interact with topoisomerase II, although the molecule acquired new properties and at lower concentration affected microtubules [16]. This work shows clearly that C4'-substituted genistein derivatives do not induce any DNA lesions, there is no ATM kinase activation, which occurs in genistein treated cells, no increase of micronucleated cells above the control level, and no DNA damage revealed with comet assays or histone γ H2A.X staining. Our results are a proof of concept, supporting previous observation that hydroxyl group at C4' of genistein is crucial for inhibitory activity of a molecule against topoisomerase II. The phenolic group at B ring was suggested to be critical for the compound to act as a traditional, redox-independent, topoisomerase II poison [40]. Substitution of the C4'-OH with a methoxy group (biochanin A) abrogates the activity of a molecule [41]. It is notable, however, that the requirement for this hydroxyl group is not absolute, because diosmetin which contains a

methoxy group in the 4'-position induce moderate levels of DNA cleavage [41].

Although the results are still too fragmentary to give comprehensive mechanistic explanation of activity of C4'-substituted genistein derivatives, we can with high probability exclude the induction of DNA double-strand breaks. The observation of autophagy induction would rather suggest that cell-cycle progression is stopped by insufficient supply with nutrients. In cells under metabolic stress the progression into S phase is stopped and, after prolonged starvation, the process of self-eating, called autophagy, is induced [32]. Our results indicate that C4'-substituted derivatives of genistein induced autophagy manifested as characteristic vesicular pattern of LC3 protein, followed by remarkable decrease of a cytoplasm volume. The tested genistein derivatives promote catabolic processes, but the mechanism of that process remains to be solved. The pattern of induction of cell-cycle-related proteins: p53, p21 and MDM-2, is strikingly similar to the one induced by AICAR [23]; the AMP mimetic compound, suggests, that new genistein derivatives may modulate functioning of the cell-cycle machinery through nutrition-sensing pathways.

Regardless the unknown yet mechanism, the C4'-substituted compounds may be useful as non-genotoxic compounds lowering the rate of proliferation of cancer cells.

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