Rosmarinic Acid Influences The Expression of Glycoforms in DLD-1 and HT-29 Colon Cancer Cells

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Article

Rosmarinic Acid Influences The Expression of Glycoforms in DLD-1 and HT-29 Colon Cancer Cells

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Abstract: Rosmarinic acid (RA) is polyphenolic compound with beneficial, health-promoting effects. Due to limited studies regarding the influence of RA on colon cancer cells, we examined how this acid influences selected glycoforms, enzymes participating in their formation and Gal-3, Akt, p53 in DLD-1 and HT-29 colon cancer cell lines. To determine the expression of studied factors, qRT-PCR, Western blotting and ELISA tests were applied. We revealed inhibitory effect of RA in both cell lines on MUC1, ppGalNAcT2, ST6GalNAcT1, C1GalT1, Cosmc, ST3GalT1, FUT3/4, Gal-3, and Akt mRNAs. p53 mRNA was increased. Moreover, we observed the suppression of Tn, T antigen as well as C1GalT1 and pAkt protein expression as the result of RA action. Apart from that, in HT-29 colon cancer cells, rosmarinic acid inhibited MUC1 extracellular domain, sialyl Tn, sialyl T, fucosylated antigens, and Gal-3 expression. p53 protein was elevated upon RA action. In DLD-1 cancer cells, RA diminished the expression of sialyl Tn in culture medium and sialyl T in cell lysates. The data suggest potential usefulness of RA as a complementary agent supporting chemotherapy in colon in cancer treatment.

Keywords: colon cancer; glycosylation; MUC1; rosmarinic acid

1. Introduction

Colon cancer is estimated to be one of the most dangerous malignant tumors which often spreads to the lungs, ovaries, liver and other parts of the gastrointestinal system, and also is the world's second notable cause of cancer related deaths. According to GLOBOCAN 2020 there will be about 1.92 million new cases of colon cancer worldwide in 2040 [1–4]. Surgery and chemotherapy are the main tools of this cancer therapy, with much higher effectiveness at early stages of the disease. However, such treatment is very often linked to high cytotoxicity of chemotherapeutics as well as resistance to healing [2]. New therapies are introduced, but 5-year relative survival still remains at only about 65% [5]. An increasing number of reports shows that natural medications produced from plants, used by about 70% of the world population, may be applied in the healing of many cancers, including colon ones [2,6]. It has been demonstrated that polyphenolic compounds, characterized as derivatives of 2-phenyl-benzo-γ-pyrone, extracted from many herbs, are responsible for anti-cancer effects through induction of apoptosis and autophagy, cell cycle arrest, modulation of tumor-suppressive microRNA or by triggering different signaling pathways [6,7]. Using them as potential anti-cancer agents is especially important because of their very few side effects, unlike many chemotherapeutics.

The impact on specifically altered glycoforms of cancer cells, inherently associated with the development of tumors, can be one more, less explored goal of bioactive molecules. Alterations in mucin-type O-linked glycosylation have been related to cancer development and poor prognosis [8–10]. Glycosylation-mediated progress of cancer is based on mediation of glycans in oncogenic signal transduction, ligand-receptor interaction, cell-cell, and cell-matrix adhesion in tumor cells [11].

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Cancer cells exhibit a considerable range of glycosylation modifications compared to normal ones [12]. Alterations in glycosylation allow malignant cells to stimulate cell mobility, cell adhesion, receptor activation, and by that contributing to the invasive phenotype [13]. Transmembrane MUC1 mucin is one of the main carriers of tumor associated glycoforms. It is reported that MUC1 has minimal or even absent expression in healthy colonic tissues while it is upregulated in about 55 – 100% of colorectal cancers [14,15]. Aberrant, tumor-associated carbohydrate antigens (TACAs), presented especially by cancerous MUC1, include prematurely terminated monosaccharides (Tn antigens) or disaccharides (T antigen), their sialylated forms (sialyl Tn and sialyl T) as well as Lewis antigens [12].

Rosmarinic acid (RA), an ester of caffeic acid and 3-(3,4-dihydroxyphenyl)lactic acid (Figure 1), polyphenol present in medicinal plants, herbs, and species, is a compound with many beneficial, health-promoting effects, including anti-cancer [16,17]. RA has been reported to act by different mechanisms such as reduction of TNF-α, COX-2, IL-6 levels, modulation of p65 expression, stimulation apoptosis by modifying various gene expression, and involvement in apoptosis regulation [18,19]. Recently we have reported promising results revealing potential of rosmarinic acid against AGS gastric cancer cells. RA was able to suppress expression of MUC1 mucin, specific cancer-related carbohydrate antigens, enzymes responsible for their formation as well as some other factors responsible for cancer development [20,21]. Upon these encouraging outcomes we decided to examine the action of rosmarinic acid towards glycoforms of DLD-1 and HT-29 colon cancer cells. According to our knowledge there are no reports considering such effects.

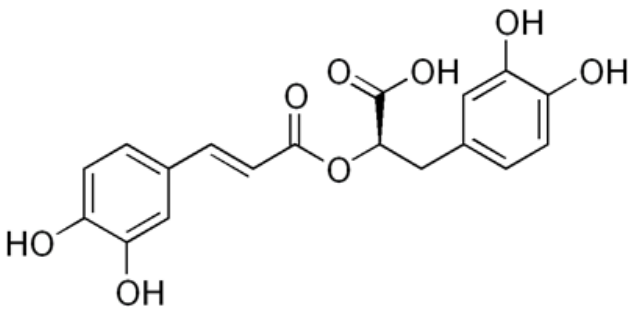

Figure 1. T Structure of rosmarinic acid.

2. Results

2.1. Viability of DLD-1 and HT-29 Colon Cancer Cells in the Presence of Rosmarinic Acid

MTT test was applied to determine the effect of rosmarinic acid action on DLD-1 and HT-29 colon cancer cells. It was revealed that 50 – 400 μM concentrations of RA exerted very low cytotoxicity on examined cells (Figure 2). Viability of the cells was not lower than 80% compared with control where no acid was added. In our experiments we decided to choose 200 and 400 μM RA concentration.

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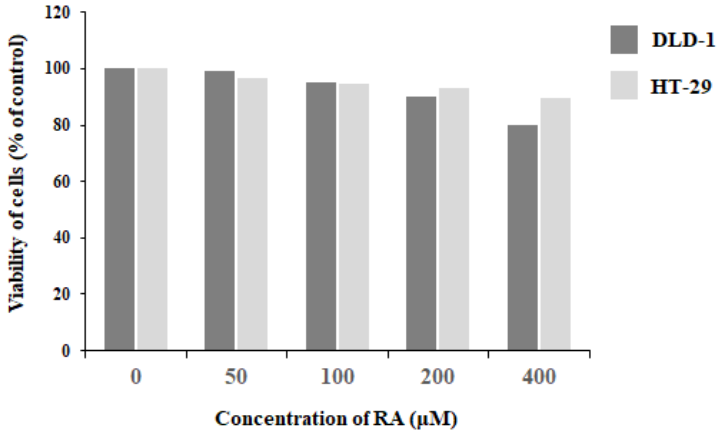

Figure 2. Viability of DLD-1 and HT-29 colon cancer cells treated for 24 h with 0 – 400 μM concentrations of rosmarinic acid. Mean values ±SD are the mean of triplicate culture.

2.2. The Effect of Rosmarinic Acid on MUC1 Expression

As MUC1 is the main carrier of sugar antigens examined in the study, at first, we decided to check the influence of RA on this mucin. The expression of MUC1 mRNA as well as the level of extracellular domain of this glycoprotein culture medium were assessed. Quantitative real time PCR (qRT-PCR) revealed inhibitory effect of RA on MUC1 mRNA in both examined cancer cells, with stronger result in DLD-1 cell line (Figure 3A). However, inhibitory effect of rosmarinic acid on MUC1extracellular domain released to the culture medium is seen only in DLD-1 cells (Figure 3B).

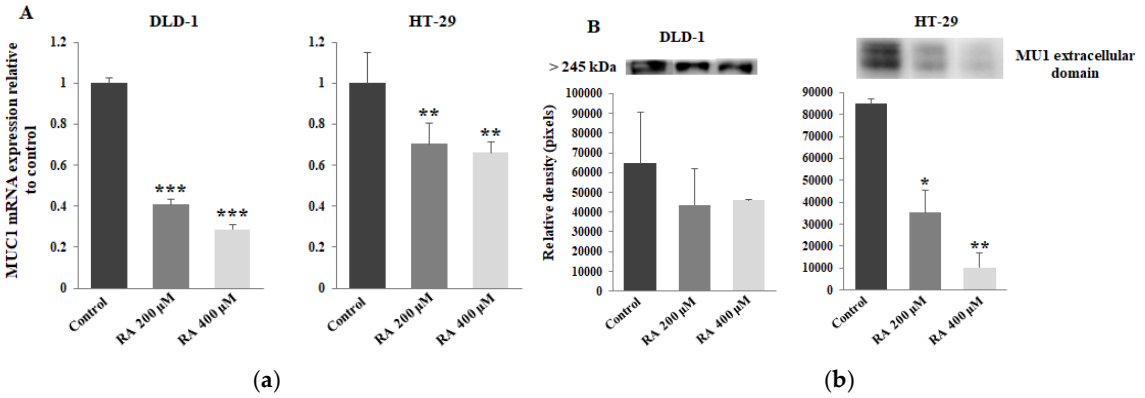

Figure 3. The effect of rosmarinic acid on MUC1 mRNA and MUC1 extracellular domain expression in DLD-1 and HT-29 colon cancer cells. The cells were incubated for 24 h with 200 and 400 μM RA. mRNA was determined by qRT-PCR (**A**). The results are presented as a relative fold change in mRNA expression of gene in comparison to the gene in control, where expression was set at 1. ± SD are the mean of triplicate cultures. $^{**}p < 0.01$, $^{***}p < 0.001$. MUC1 extracellular domain in culture medium was assessed by Western blotting (**B**). The intensities of the bands were quantified by densitometric study. Data represent the mean ± SD of triplicate culture. $^{*}p < 0.05$, $^{**}p < 0.01$ compared to control.

2.3. The Effect of RA on ppGalNAcT2, ST6GalNAcT1, Tn, and Sialyl Tn Antigens Expression

Tn antigen (GalNAcα-Ser/Thr) and its sialyl form (sTn; Neu5Acα2-6GalNAcα-Ser/Thr) are the results of the action of proper polypeptide α-N-acetylgalactosaminyltransferase (ppGalNAcT) and sialyltransferase (ST6GalNAcT), respectively. qRT-PCR assays demonstrated that RA with 200 and 400 μM concentration effectively suppressed the expression of ppGalNAcT2 mRNA in both cell lines (Figure 4A). However, the examination of Tn antigen by ELISA test with biotinylated VVA lectin didn't reveal such inhibitory effect in DLD-1 cell line (Figure 4B). In case of HT-29 colon cancer cells, suppression of Tn antigen was confirmed in cell lysates, as the effect of 400 μM RA action and in

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culture medium as the effect of two applied concentrations of acid (Figure 4C). RA with both concentrations decreased the expression of ST6GalNAcT1 on mRNA level in DLD-1 cells, but in case of HT-29 line, such effect was revealed only with higher concentration of rosmarinic acid (Figure 4D). In ELISA test for DLD-1 cell line, performed with biotinylated SNA lectin detecting sialyl Tn antigen, inhibitory effect of RA was observed only in culture medium (Figure 4E). In HT-29, such suppression was revealed in culture medium as well as in cell lysates only by 400 μM rosmarinic acid (Figure 4F).

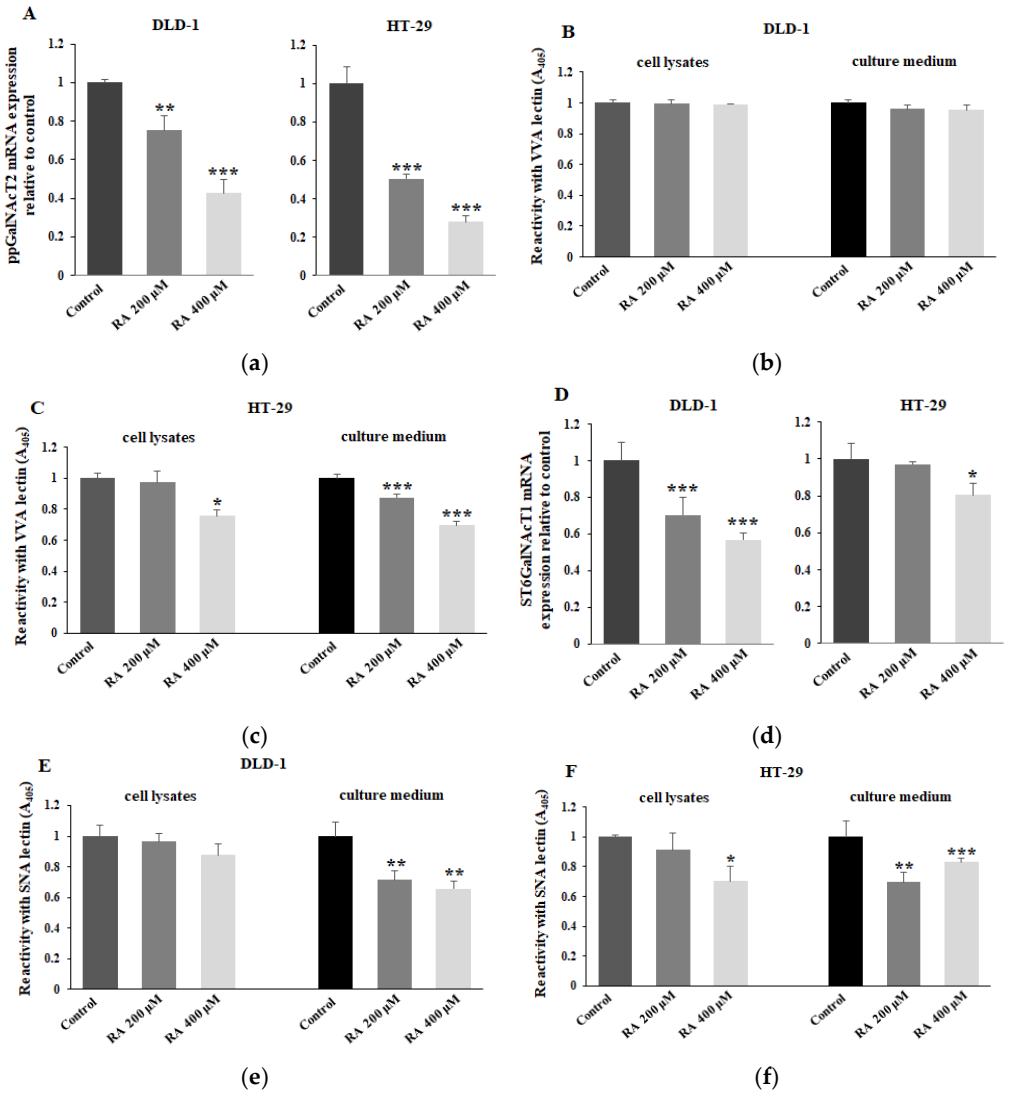

Figure 4. The effect of RA on ppGalNAcT2 mRNA, ST6GalNAcT1 mRNA, Tn and sialyl Tn antigen expression in cell lysates and culture medium. DLD- and HT-29 colon cancer cells were incubated for 24 h with 200 and 400 μM rosmarinic acid. ppGalNAcT2 mRNA (**A**) and ST6GalNAcT1 mRNA (**D**) were assessed by qRT-PCR. The results are presented as a relative fold change in mRNA expression of gene in comparison to the gene in control, where expression was set at 1. ± SD are the mean of triplicate cultures. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Tn and sialyl Tn antigens relative expressions were determined by ELISA tests with specific, biotinylated lectins. VVA was applied to assess Tn antigen (**B,C**) and SNA was used to assess sialyl Tn (**E,F**). The results are expressed as absorbance at 405 nm after reactivity with lectins. Values ± SD are the mean from three independent analysis. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to untreated control.

2.4. The Effect of RA on C1GalT1, Cosmc, ST3GalT1, T, and Sialyl T Antigens Expression

T (TF) sugar structure (Galβ1-3GalNAcα-Ser/Thr) and sialyl T (Neu5Acα2-3Galβ1-3GalNAcα-Ser/Thr) are formed upon action of core 1 β1-3galactosyltransferase (T synthase; C1GalT1) and β-galactoside α2-3sialyltransferase (ST3GalT1), respectively. T synthase needs unique molecular

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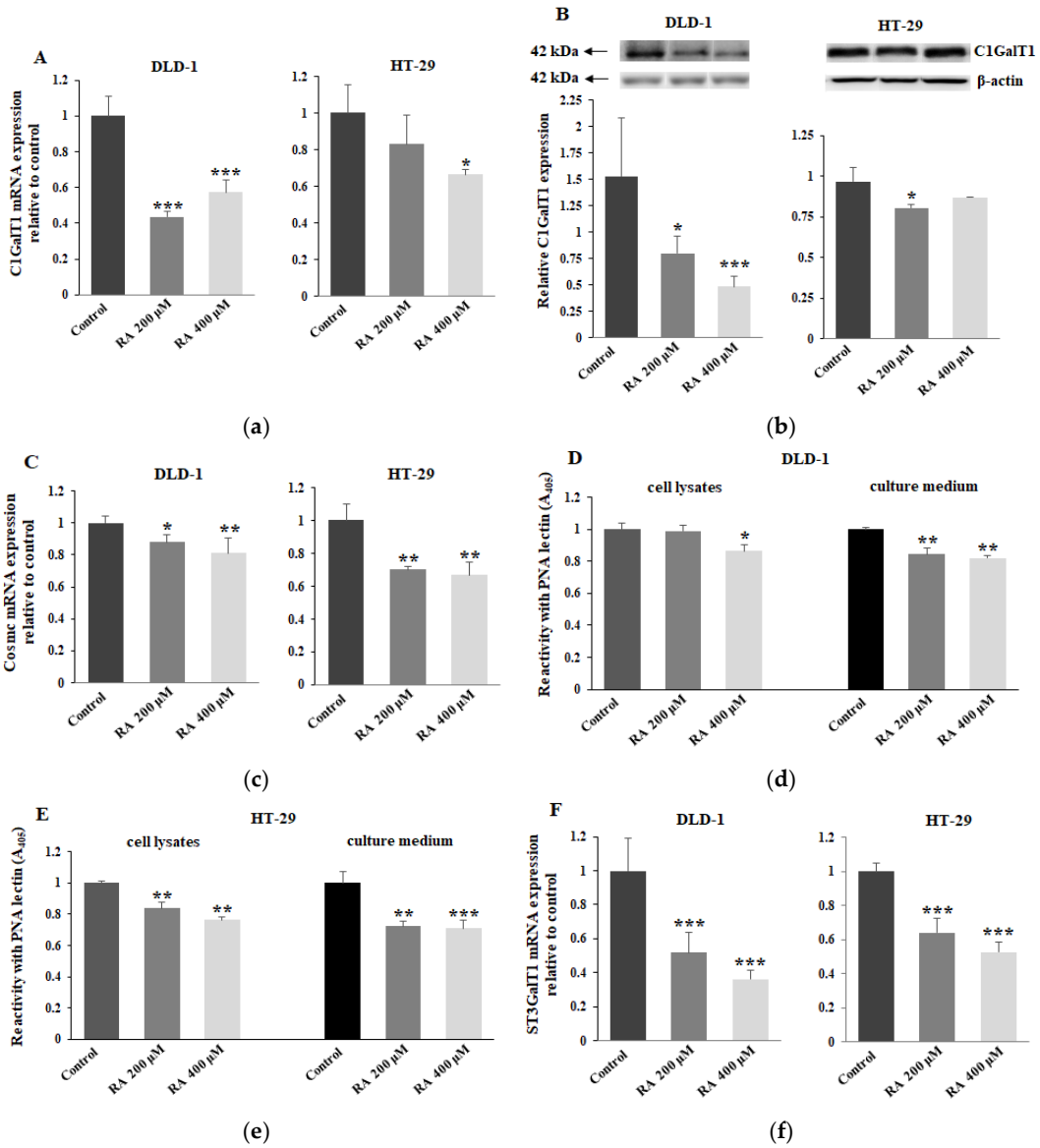
endoplasmic reticulum chaperone (Cosmc; C1GalT1C1) which is required to synthetize active form of this enzyme. C1GalT1 mRNA was significantly suppressed by the action of both RA concentrations in DLD-1 cells, and by 400 μM in HT-29 cells (Figure 5A). Similar inhibitory effect was revealed for the enzyme on the protein level in DLD-1 cells; in case of HT-29, only as the result of 200 μM RA action (Figure 5B). Cosmc mRNA in both cancer cells was suppressed by both concentrations of RA (Figure 5C). We revealed also inhibition of T antigen expression, in ELISA test with biotinylated PNA lectin, by both concentrations of RA in culture medium of DLD-1 cells, and by 400 μM RA in cell lysates (Figure 5D). In HT-29 colon cancer cells, suppression of this antigen expression was observed in cell lysates and culture medium by both RA concentrations (Figure 5E). ST3GalT1 on mRNA level was inhibited in two cell lines by two applied doses of rosmarinic acid (Figure 5F). In ELISA test with biotinylated MAAII lectin we revealed that RA suppressed sialyl T antigen expression in cell lysates of DLD-1 cells (Figure 5G), and in cell lysates and culture medium of HT-29 cells (Figure 5H).



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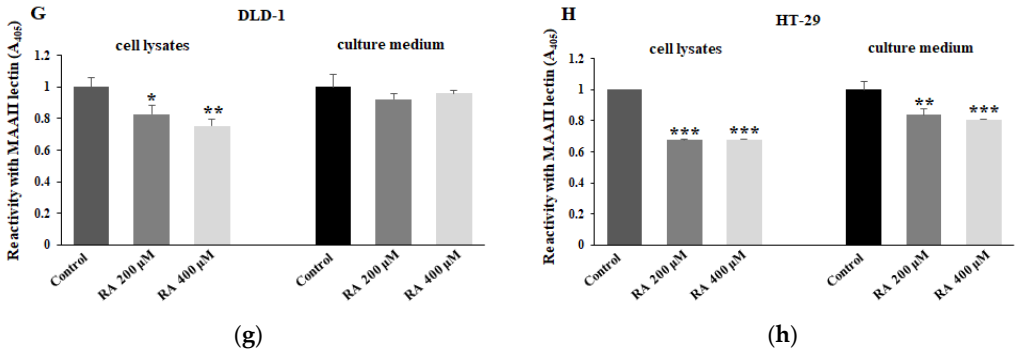
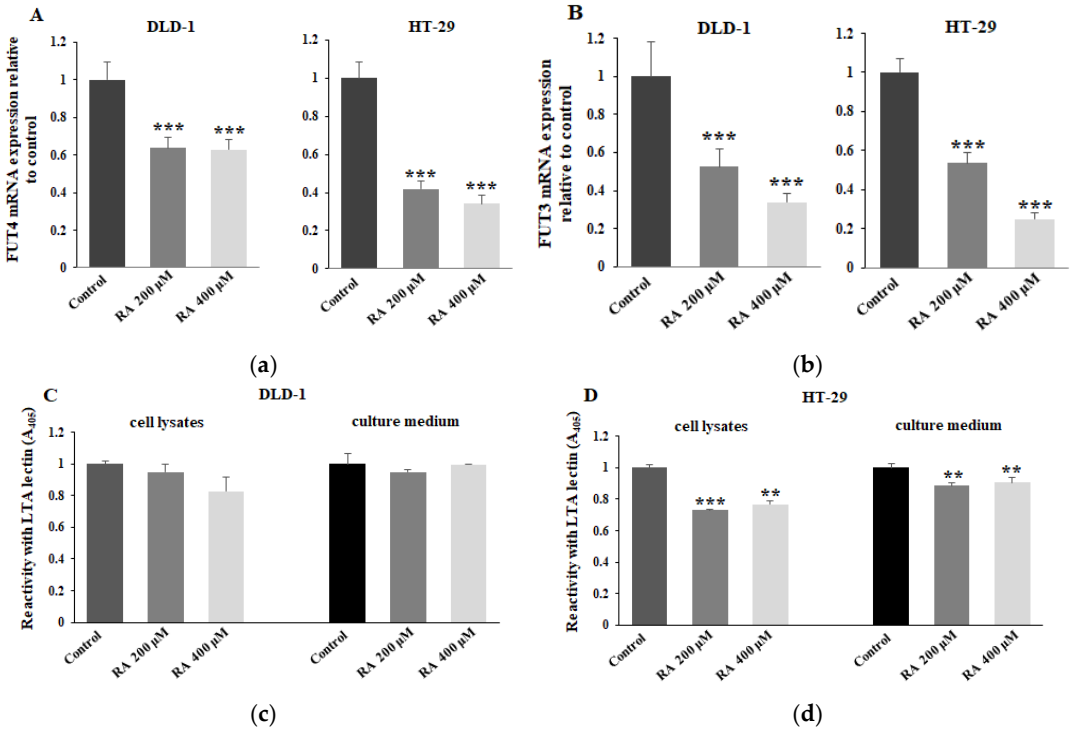


(**g**) (**h**)

Figure 5. The effect of rosmarinic acid on C1GalT1 mRNA, C1GalT1 protein, Cosmc mRNA, ST3GalT1 mRNA, T and sialyl T antigens in cell lysates and culture medium of DLD-1 and HT-29 colon cancer cells. The cancer cells were incubated for 24 h with 200 and 400 μM RA. C1GalT1 (**A**), Cosmc (**C**), and ST3GalT1 (**F**) mRNAs were determined by qRT-PCR. The results are presented as a relative fold change in mRNA expression of gene in comparison to the gene in control, where expression was set at 1. ± SD are the mean of triplicate cultures. *$p <$ 0.05, **$p < 0.01$, ***$p < 0.001$. C1GalT1 protein (**B**) was assessed by Western blotting. β-actin served as a protein loading control. The bands were analyzed by densitometric study. Data represent the mean ± SD of triplicate culture. *$p < 0.05$, ***$p < 0.001$ compared to control. T and sialyl T antigens expressions were determined by ELISA tests with biotinylated lectins (PNA for T antigen (**D,E**) and MAAII for sialyl T (**G,H**)). The results are expressed as absorbance at 405 nm after reactivity with lectins. Values ± SD are the mean from three independent analysis. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to untreated control.

2.5. The Effect of RA on FUT3, FUT4, and Fucosylated Antigens Expression

FUT3 and FUT4 are fucosyltransferases responsible for fucose addition to Gal or GlcNAc and Lewis antigens formation. FUT3 and FUT4 on mRNA level were suppressed by rosmarinic acid in two colon cancer cell lines (Figure 6A,B). However, in ELISA tests, Lewis antigens recognized by LTA lectin were not influenced by RA in DLD-1 cell lines, and inhibited in HT-29 cells (Figure 6C,D).



(**a**) (**b**) (**c**) (**d**)

Figure 6. The effect of rosmarinic acid on FUT3, FUT4 mRNAs and Fucα1,3-GalNAc antigen. DLD-1 and HT-29 colon cancer cells were incubated for 24 h with 200 and 400 μM RA. FUT3 (**A**) and FUT4 (**B**) mRNAs were assessed by qRT-PCR analysis. The results are presented as a relative fold change in mRNA expression of gene

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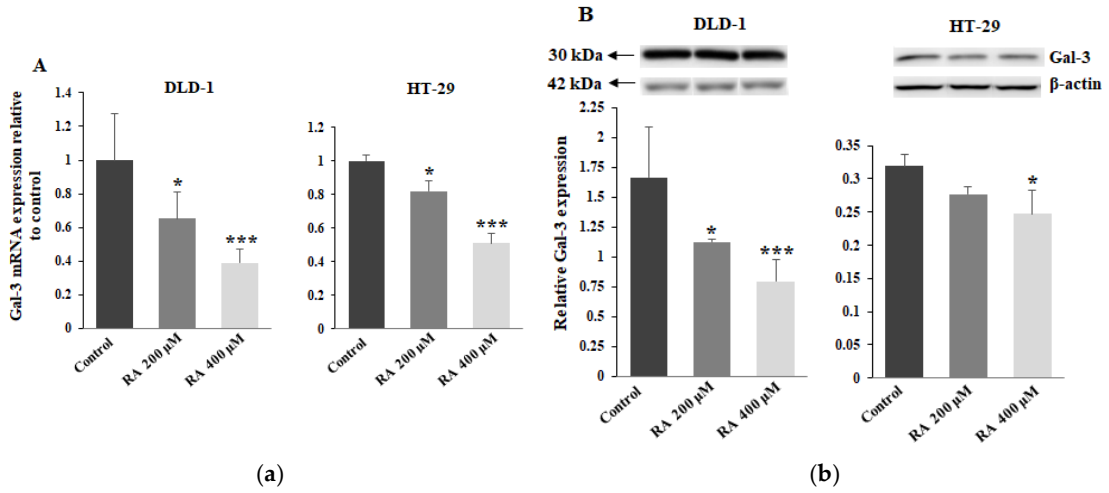
in comparison to the gene in control, where expression was set at 1. ± SD are the mean of triplicate cultures. ***p < 0.001. Fucα1,3-GalNAc antigen expression in cell lysates and culture medium was determined by ELISA test with biotinylated LTA lectin (**C,D**). The results are expressed as absorbance at 405 nm after reactivity with lectin. Values ± SD are the mean from three independent analysis. **p < 0.01, ***p < 0.001 compared to untreated control.

2.6. The Effect of RA on Gal-3 Expression

Gal-3 is multifunctional, oncogenic protein with high affinity to T carbohydrate antigen. Both applied concentrations of rosmarinic acid suppressed Gal-3 mRNA expression in two colon cancer cell lines (Figure 7A). Moreover, Gal-3 protein expression was inhibited by 200 and 400 μM RA in DLD-1 cells and by 400 μM RA in HT-29 cells (Figure 7B).

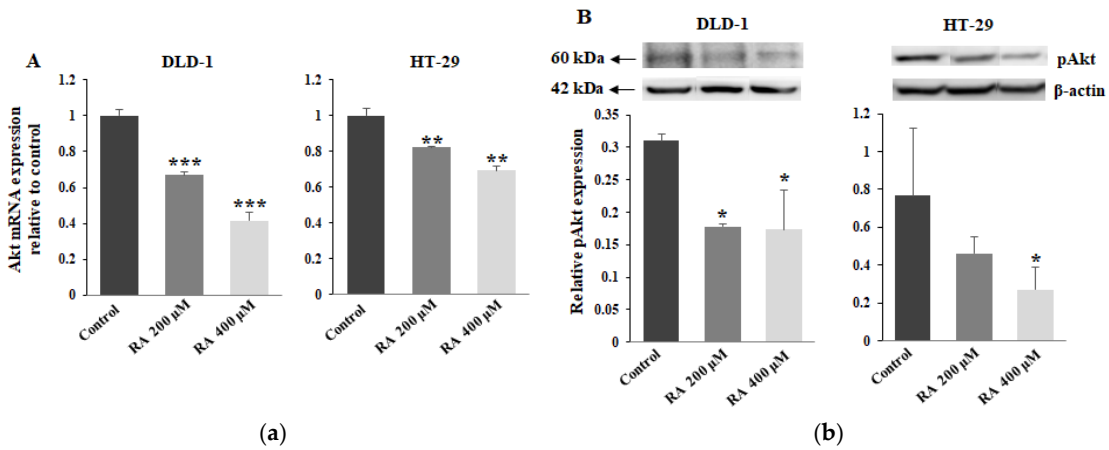


(**a**) (**b**)

Figure 7. The effect of rosmarinic acid on Gal-3 mRNA and Gal-3 protein expression in DLD-1 and HT-29 colon cancer cells. Cancer cells were incubated for 24 h with 200 and 400 μM RA. Gal-3 mRNA (**A**) was assessed by qRT-PCR analysis. The results are presented as a relative fold change in mRNA expression of gene in comparison to the gene in control, where expression was set at 1. ± SD are the mean of triplicate cultures. *p < 0.05, ***p < 0.001. Gal-3 protein in cell lysates (**B**) was determined by Western blotting. β-actin served as a protein loading control. The bands were analyzed by densitometric study. Data represent the mean ± SD of triplicate culture. *p < 0.05, ***p < 0.001 compared to control.

2.7. The Effect of RA on Akt Expression

Akt is protein kinase playing critical role in promoting cell survival and apoptosis. Rosmarinic acid inhibited Akt mRNA in both colon cancer cell lines (Figure 8A). In addition, active, phosphorylated form of Akt was suppressed by both concentrations of RA in DLD-1 cells as well as in HT-29 after action of 400 μM RA (Figure 8B).



(**a**) (**b**)

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Figure 8. The effect of RA on Akt mRNA and pAkt protein expression in DLD-1 and HT-29 colon cancer cell lines. Cancer cells were incubated for 24 h with 200 and 400 μM RA. Akt mRNA (**A**) was analyzed by qRT-PCR analysis. The results are shown as a relative fold change in mRNA expression of gene in comparison to the gene in control, where expression was set at 1. ± SD are the mean of triplicate cultures. $^{**}p < 0.01$, $^{***}p < 0.001$. pAkt in cell lysates (**B**) was determined by Western blotting. β-actin served as a protein loading control. The bands were assessed by densitometric study. Data represent the mean ± SD of triplicate culture. $^{*}p < 0.05$, compared to control.

2.8. The Effect of RA on p53 Expression

In Figure 9A we demonstrate that p53 mRNA, tumor suppressor protein, was stimulated by 200 μM RA concentration in both colon cancer cell lines. 400 μM concentration of rosmarinic acid didn't influence this factor in DLD-1 cells, and surprisingly inhibited p53 in HT-29 cells. p53 protein expression was stimulated only in HT-29 cells by lower concentration of RA, and inhibited by higher concentration of acid (Figure 9B).

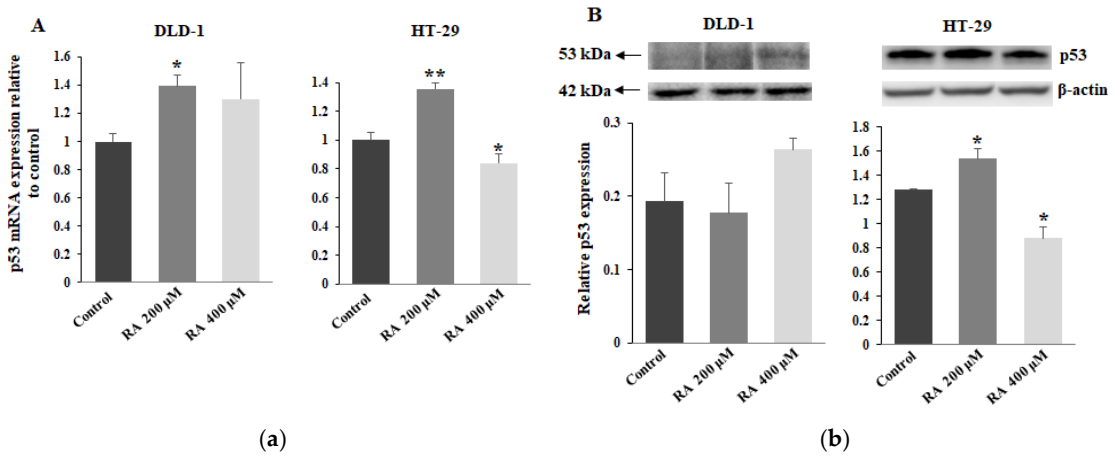

Figure 9. The effect of RA on p53 mRNA and protein expression in DLD-1 and HT-29 colon cancer cell lines. The cells were incubated for 24 h with 200 and 400 μM RA. p53 mRNA (**A**) was analyzed by qRT-PCR. The results are shown as a relative fold change in mRNA expression of gene in comparison to the gene in control, where expression was set at 1. ± SD are the mean of triplicate cultures. $^{*}p < 0.05$, $^{**}p < 0.01$. p53 in cell lysates (**B**) was determined by Western blotting. β-actin served as a protein loading control. The bands were assessed by densitometric study. Data represent the mean ± SD of triplicate culture. $^{*}p < 0.05$, compared to control.

3. Discussion

It has been revealed that altered glycosylation is a common feature in various steps of malignant transformation and progression. Very often glycosylation variations are relatively specific for the type and stage of cancer, thereby making glycans as potential cancer biomarkers as well as targets for anticancer therapy [10–12,22]. There are many ways by which O-glycans can regulate tumorigenesis. Carbohydrates on a receptor protein tend to affect its properties, activity, expression level, and stability. Glycans can directly mediate oncogenic signal transduction, ligand-receptor interactions, cell-cell, and cell-matrix adhesion in malignant cells, and in this way are associated with cancer progression and metastasis which correlate with poor prognosis and high mortality [11,12,23,24]. There are also reports concluding, that expression of specific glycans is associated with tumor escape from immune defenses [25,26].

MUC1 is main, heavily O-glycosylated protein of gastrointestinal epithelium, overexpressed and associated with progression in diverse cancers, such as colon, breast, lung, pancreatic, and prostate [14,23,24,27–31]. Thus, glycosylation of epithelium origin cancer cells is said to be mucin-type O-linked glycosylation [9,11,22]. MUC1 is considered as oncogene that regulates cell growth,

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proliferation, metastasis, apoptosis by participating in various signaling pathways [32]. Moreover, aberrantly glycosylated MUC1 can be recognized by B cell-derived immunoglobulins and afterwards evokes tumor-specific adaptive immune feedback by the activation of tumor antigen-specific cytotoxic T lymphocytes [33]. Thus, cancer-derived O-glycan signatures have been highlighted as potential tumor-specific therapeutic targets, as well as biomarkers for clinical outcome and therapeutic response [10]. Main altered glycosylation features include: hypoglycosylation and exposition of specific tumor-associated carbohydrate antigens (TACAs), like prematurely terminated, short Tn or T antigens, their sialylated forms as well as fucosylated Lewis antigens [8,9,23,27,34,35]. Such truncated O-glycans are observed especially at the earliest stages of cellular malignant transformation [29]. The accurate molecular mechanism by which abnormal glycosylation is able to induce adverse metabolic and cellular signaling leading towards tumor progression, is still a subject of survey.

Recently, an emphasis has been put down on examining the effects of various natural compounds at different steps of cancer development. One of such compounds is rosmarinic acid, plant polyphenol, frequently found in Laminaceae and Boraginaceae families [36]. There are some reports revealing anti-cancer action of RA in colon cancer cells [37]. It has been demonstrated that rosmarinic acid induced apoptosis, inhibited metastatic potential, regulated EMT, suppressed MMPs, mainly by AMPK activation [38], by p38 MAPK/AP-1 signaling [39] or by inhibiting ERKs phosphorylation [40–42]. Other authors evidenced anti-inflammatory action of RA by inhibition of the expression of COX-2 [43,44]. Targeting Warburg metabolism by rosmarinic acid has been also indicated as promising action of this acid in colon cancer treatment [45,46]. However, to date, very little attention has been given to the influence of rosmarinic acid on glycoforms of colon cancer cells. Thus, the examining the impact of RA on specific carbohydrate structures in HT-29 and DLD-1 colon cancer cells, was the main aim of our study. Our concept was based on formerly received, promising results concerning anti-cancer action of rosmarinic acid in AGS gastric cancer cells [20,21].

In the presented study we revealed that rosmarinic acid had potential to inhibit MUC1 expression as well as some specific cancerous glycoforms in colon cancer cells DLD-1 and HT-29. We postulate that such action of RA can be considered as kind of therapeutic approach.

It has been reported that truncated Tn and sialyl Tn antigens are highly expressed in colon carcinomas. They appear to be colon-cancer associated antigens acting as markers of poorly differentiated cancers [34,47]. Yamamoto et al. [48] have been suggested that Tn carbohydrate antigen overexpression directly promoted oncogenic hallmark including enhanced cell proliferation, decreased apoptosis, increased adhesion as well as migration capacities. The authors have reported recently that enhanced Tn expression coincided with intensified tumor growth, particularly at later steps of tumor development. Sialyl-Tn antigen has been also described to promote tumor progression. In gastric cancer cells, this epitope has been reported to modulate cell cycle, apoptosis, adhesion, cell-cell aggregation and invasion [48,49]. Apart from that, sialyl Tn antigen in bladder cancer, has been described to be related with high-grade tumors, invasion, poor survival, tumor dissemination and metastasis [50]. We revealed that RA had the potential to inhibit enzymes responsible for Tn and sialyl Tn antigens formation, ppGalNAcT and ST6GalNAcT mRNA, respectively. However, the expression of Tn antigen was suppressed mainly in HT-29 cells in culture medium.

T antigen, highly expressed in carcinomas, is associated with invasiveness, tumor growth, and high metastatic potential. It is said to play a key role in the adhesion of cancer cells to the endothelium via interaction with galectin-3, and by that promoting metastasis [51]. It has been reported that T antigens and Gal-3 are positively associated with colon cancer metastasis [11,52]. In colon cancer, MUC1 is often decorated with T epitopes [53]. It has been revealed that association between Gal-3 and T antigen on MUC1 can lead to EGFR related carcinogenesis, cancer progression as well as cell homotypic aggregation what results in promoting of cancer cells survival by inhibition of cellular anoikis [25]. C1GalT1, favored by molecular chaperone COSMC, is enzyme responsible for T antigen formation. It is considered as the critical enzyme implicated in O-glycans synthesis [11,54]. C1GalT1

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expression is upregulated in most cancers, what is firmly associated with malignant behavior of tumors, including proliferation, invasion or tumor spread [55]. Our results concerning T and Tn antigens, as well as enzymes responsible for their production, prove the potential of rosmarinic acid in anti-cancer therapy, as we revealed the suppression of the most mentioned factors.

The next, crucial in tumorigenesis carbohydrate structures are the blood group fucosylated Lewis antigens. Fucose containing glycans and enzymes implicated in fucosylation process are involved in gaining the functional features of cancer cells such as unlimited cell progression, tissue invasion, metastasis or immune evasion [56]. It has been reported that such fucosylated antigens can serve as ligands for e.g., E-, P-selectins of vascular endothelial cells and as result of such potential interactions, could promote extravasation and metastasis of cancer cells [12,21]. In our study we demonstrate inhibitory effect of rosmarinic acid on FUT3 and FUT4 in both examined cell lines as well as Lewis structures in HT-29 cells. Such action of RA allows to treat it as attractive candidate for anticancer agent.

Inhibiting of Akt expression and stimulating of p53 by RA also confirm its validity in cancer treatment. It is well documented that overexpression of phosphorylated Akt is among major factors initiating tumor development and allowing resistance to the conventional chemotherapy in various cancers [57]. p53, a powerful tumor suppressor is considered as a crucial barrier against cancer initiation and progression. It reveals its powerful action by controlling the cell cycle, apoptosis, DNA repair as well as angiogenesis [58]. Thus, the agents suppressing Akt, triggering p53, like rosmarinic acid, are among potential candidates in supporting of cancer treatment.

4. Materials and Methods

4.1. Cell Culture

DLD-1 (CCL-221) and HT-29 (HTB-38) human colorectal adenocarcinoma cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). DLD-1 cells were cultured in RPMI 1640 medium (ATCC, Manassas, VA, USA), and HT-29 cells in McCoy's 5a medium (Pan Biotech., Aidenbach, Lower Bavaria, Germany) at 37°C, 5% CO2 in humidified air. Both media were supplemented with Fetal Bovine Serum (10%) (FBS; Gibco, Waltham, MA, USA), streptomycin (100 µg/mL), and penicillin (100 U/mL) (Sigma, St. Louis, MO, USA). Cells were seeded into 6-well plates in growth medium (1 mL), grown to 70% confluency, and then used for the further analyses. Dimethyl sulfoxide (DMSO; Sigma, St. Louis, MO, USA) was applied to dissolve rosmarinic acid (Roth, Karsruhe, Germany). Stock solution of RA was 111 mM. The cells were cultured with for 24 h in proper FBS-free medium with 200 and 400 µM RA. Then the cells were washed with Phosphate Buffered Saline (PBS; Corning, Manassas, VA, USA) and lysed with RIPA buffer (Sigma, St. Louis, MO, USA) for 20 min at 4oC with Protease Inhibitor Cocktail (Sigma, St. Louis, MO, USA) diluted 1:200. Next, intense vortexing and centrifugation at 1,000 x g for 5 min at 4°C were performed. The supernatants of cell lysates and culture media were collected, frozen at -70°C and used for further analyses. BCA Protein Assay Kit (Pierce, USA) was applied for the measurements of protein concentration. For quantitative real-time PCR, the wells were washed 3-times with sterile PBS (10 mM), collected and sonified (Sonics Vibra cell; Sonics & Materials, Leicestershire, UK) (10 W, 3-times for 15 s on ice). For RNA isolation, aliquots of the homogenate were used.

4.2. Cell Viability Test

The measurement of the viability of the cultured colon cancer DLD-1 and HT-29 cells, in the presence of rosmarinic acid (50 – 400 µM), were assessed using were assayed using 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, St. Louis, MO, USA) according to the procedure of Carmichael et al. [59]. Briefly, cells were cultured in six-well plates to get 70% of confluency and incubated for 24 h with different concentration of rosmarinic acid. Next, 1 mL of MTT solution (0.5 mg MTT/mL PBS) was added to each well and incubation at 37 oC in 5% CO2 for 4 h was performed. The absorbance of the converted dye was read at 570 nm. The viability

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of colon cells with RA was determined as a percentage of control cells without RA (100% cell viability).

4.3. Western Blotting

To detect the expression of C1GalT1, Gal-3, pAkt,, p53 proteins in cell lysates and MUC1 extracellular domain in culture media, electrophoresis on polyacrylamide gels (7.5 – 13%) and Western blotting assays were carried out. The samples containing 20 μg of protein, diluted in probe sample buffer with 2.5% SDS (Sigma, St. Luis, MO, USA) were subjected to electrophoresis and transferred to on Immobilon P (Millipore, Bedford, MA, USA) according to Towbin et al. [60]. 5% skim milk in Tris Buffered Saline (TBS) supplemented with 0.05% Tween 20 (Sigma, St. Luis, MO, USA) was used to block the membranes (after Western blotting) for 1 h at room temperature (RT). Then the membranes were washed with TBS-T and incubated with specific primary antibodies (listed in Table 1) overnight at 4 oC. Proper horseradish peroxidase-conjugated secondary antibodies were applied to detect immunoreactive complexes. To visualize the protein bands, enhanced chemiluminescence procedure with Westar Hypernova, ECL substrate for Western blotting (Cyangen, Bologna, Italy) was performed. The Gene Tools program (Syngene, Frederick, MD, USA) was used to quantify densitometrically the intensity of the bands (normalized for β-actin).

Table 1. Antibodies used in electrophoresis and Western blotting.

Antibody	Clone	Source
Anti-MUC1; extracellular domain (mouse IgG)	BC2	Abcam
Anti-C1GalT1 (mouse IgG)	F-31	Santa Cruz
Anti-Gal-3 (mouse IgG)	B2C10	Santa Cruz
Anti-pAkt (rabbit IgG)	D9E	Cell Sign Tech
Anti-p53 (rabbit IgG)	7F5	Cell Sign Tech
Anti-β-actin (rabbit IgG)		Sigma
Anti-mouse IgG peroxidase conjugated		Sigma
Anti-rabbit IgG peroxidase conjugated		Sigma

4.4. ELISA

To demonstrate the relative levels of carbohydrate antigens in cell lysates and culture media, ELISA tests with specific biotinylated lectins (Vector, Burlingame, CA, USA) were applied (Table 2). The procedure was performed according to previously described [61]. Briefly, 50 μL of cell lysates or culture medium (100 μg protein/mL) were applied on Microtiter plates (NUNC F96 Maxisorp, Roskilde, Denmark) and incubated overnight at RT. Then, blocking reagent for ELISA (Roche Diagnostics, Mannheim, Germany) was used for 1h at RT. As washing buffer, PBS with 0.05% Tween (100 μL) was applied after every step. Next, the plate was incubated with biotinylated lectins diluted to 5 μg/mL (2h at RT). Then, horseradish peroxidase avidin D (Vector, Burlingame, CA, USA), with high affinity to biotin, was used (1h at RT). As the final step, 100 μL of ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma, St. Luis, MO, USA) was applied to expand the colored reaction. Absorbances were read at 405 nm after 20 – 40 min of incubation at RT. The samples were examined in triplicate.

Table 2. Biotinylated lectins used in ELISA tests.

Lectin	Specificity
VVA (*Vicia villosa* agglutinin)	Tn antigen (GalNAcα1-O-Ser/Thr)
SNA (*Sambucus nigra* agglutinin)	sialyl Tn antigen (NeuAcα2,6-Gal/GalNAc)
PNA (*Arachis hypogaea* agglutinin (peanut))	T antigen (Galβ1,3-GalNAcα1-O-Ser/Thr)
MAAII (*Maackia amurensis* agglutinin)	
LTA (*Lotus tetragonolobus* agglutinin)	

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	sialyl T antigen (NeuAcα2,3-Gal)
	Lewis structure (Fucα1,3-GlcNAc)

4.5. RNA Isolation and Quantitative Rreal-Time PCR

Total RNA was isolated applying Total RNA Mini Plus Concentrator (A&A Biotechnology, Gdansk, Poland), according to the instruction. Purity and concentration of RNA was examined spectrophotometrically (Nanodrop 2000, Thermo Scientific, Waltham, MA, USA). The same amounts (1 μg) of total RNA were submitted to reverse transcription using the SensiFASTTM cDNA Synthesis Kit (Bioline, London, UK). The reaction mixture (20 μL) contained RNA template, 1 μl of Reverse Transcriptase, 4 μl of 5xTransAmp Buffer, and DEPC-treated water. The conditions of incubation were 10 min at 25°C, 30 min at 45°C, and 5 min at 70°C were. Amplification of cDNA was carried out using SensiFAST™ SYBR Kit (Bioline, London, UK) in the thermocycler CFX96 real-time system (BioRad, Hercules, CA, USA). Reaction mixture (20 μL) contained 2 μL of cDNA template (diluted 3 times), 0.4 μL of each target-specific primer (10 μmol/L) (Genomed, Warsaw, Poland) (Table 3), 2×SensiFAST SYBR No-ROX Mix (5 μL), and DEPC-treated water. As a reference gene β2-Microglobulin was applied. The qRT-PCR parameters were as follows: 95°C for 2 min (activation of DNA polymerase), followed by 40 cycles of 10 s at 95°C (denaturation), 15 s at 60°C (annealing) and 20 s at 72°C (elongation). Each sample was examined in triplicate. The formation of the reaction products was proved by studies of their melting curves. The ΔΔCt method was used to normalize the levels of target gene transcripts to β2-Microglobulin.

Table 3. Primers applied in quantitative Real-Time PCR.

Gene	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)
MUC1	ACAATTGACTCTGGCCTTCC	CAGGTTATATCGAGAGGCTGC
C1GalT1	CAAAATACGACCCTGAAGAACC	GCATCTCCCCAGTGCTAAGTC
Cosmc	GTTTGCCTGAAATATGCTGGA	AATATGCCCAAATGCCCTAAG
ppGalNAcT2	AAGAAAGACCTTCATCACAGCAATGGAGAA	ATCAAAACCGCCCTTCAAGTCAGCA
ST3GalT1	TCGGCCTGGTTCGATGA	CGCGTTCTGGGCAGTCA
ST6GalNAcT1	ACGCAGTCCTGAGGTTTAATGG	AGTTCATCAGGCGAATGGTAGTTT
FUT3	GCCGACCGCAAGGTGTAC	TGACTTAGGGTTGGACATGATATCC
FUT4	AAGCCGTTGAGGCGGTTT	ACAGTTGTGTATGAGATTTGGAAGCT
Gal-3	GCAGACAATTTTTCGCTCCATG	CTGTTGTTCTCATTGAAGCGTG
Akt	ACTGTCATCGAACGCACCTT	CTCCTCCTCCTCCTGCTTCT
p53	GTTCCGAGAGCTGAATGAGG	TCTGAGTCAGGCCCTTCTCT
β2-Microglobulin	TTTCTGGCCTGGAGGCTATC	CATGTCTCCATCCCACTTAACT

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org. Figure S3B: The effect of rosmarinic acid on MUC1 extracellular domain expression – original images; Figure S5B: The effect of RA on C1GalT1 protein expression – original images; , Figure S7B: The effect of rosmarinic acid on Gal-3 protein expression – original images; Figure S8B: The effect of RA on pAkt expression – original images; Figure S9B: The effect of RA on p53 protein expression – original images.

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