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Ganoderma Lucidum induces oxidative DNA damage and enhances the effect of 5-Fluorouracil in colorectal cancer *in vitro* and *in vivo*

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ABSTRACT

The first-line chemotherapy of colorectal cancer (CRC), besides surgery, comprises administration of 5-Fluorouracil (5FU). Apart from cytotoxic effect on cancer cells, 5FU may also cause adverse side effects. *Ganoderma Lucidum* (GLC) is a mushroom used in Traditional Eastern Medicine. We propose that natural compounds, particularly GLC extracts, may sensitize cancer cells to conventional chemotherapeutics. This combination therapy could lead to more selective cancer cell death and may improve the response to the therapy and diminish the adverse effects of anticancer drugs.

Here we demonstrate that GLC induced oxidative DNA damage selectively in colorectal cancer cell lines, whereas it protected non-malignant cells from the accumulation of reactive oxygen species. Accumulation of DNA damage caused sensitization of cancer cells to 5FU resulting in improved anticancer effect of 5FU. The results obtained in colorectal cell lines were confirmed in *in vivo* study: GLC co-treatment with 5FU increased the survival of treated mice and reduced the tumor volume in comparison with group treated with 5FU alone.

Combination of conventional chemotherapeutics and natural compounds is a promising approach, which may reduce the effective curative dose of anticancer drugs, suppress their adverse effects and ultimately lead to better quality of life of CRC patients.

1. Introduction

Colorectal cancer (CRC) is the third most common type of cancer in the world and the second leading cause of cancer-related deaths in Europe with the highest incidence in Central Europe. With estimated 694 000 deaths per year [1] and with assumed increase by 77% in the number of newly diagnosed CRC cases in 2030, CRC represents a serious health, social and economic problem [2].

Conventional chemotherapeutic treatment of CRC is based on the 5-Fluorouracil (5FU); in the monotherapy or in a combination with irinotecan or platinum derivatives. As the patient's prognosis is primarily determined by the stage of the disease, 5FU based therapy is the standard therapeutic scheme for CRC in stage II and III [3]. Nevertheless, an overall response rate to 5FU monotherapy in more advanced CRC is limited to 10–15% [4]. Combination of 5FU with other cytotoxic agents would not only improve the response to therapy but also reduce the undesirable reaction to these drugs [5,6]. The most common adverse effects of 5FU comprise: nausea, vomiting, diarrhea, mucositis of the

Abbreviations: 5FU, 5-Fluorouracil; CRC, colorectal cancer; CTRL, control; DDR, DNA damage response; DMSO, dimethyl sulfoxide; GLC, *Ganoderma Lucidum*; H_2DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; PI, Propidium iodide; ROS, Reactive oxygen species; SDS, Sodium dodecyl sulfate

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oral cavity (mucosal and submucosal tissue damage), headache, skin pruritus, myelosuppression (suppression of hematopoietic function of the bone marrow, leukopenia, pancytopenia and thrombocytopenia), anemia, cardiotoxicity, agranulocytosis, alopecia (hair loss), photosensitivity, hand-foot syndrome, depression and anxiety [7]. The administration of 5FU in combination with folic acid (leucovorin) enhances patients' survival by approximately 10–15%. Therapy regimen combining 5FU with oxaliplatin and leucovorin (FOLFOX) add additional 7% to 3-year disease-free survival in comparison with the scheme without oxaliplatin [8]. Another possible way to improve conventional therapy may be, among others, targeting DNA damage response (DDR) pathways [9]. DDR plays an essential role in the elimination of DNA damage, thereby preventing cells from genomic instability and malignant transformation. On the other hand, DDR is also involved in patients' response to therapy. DNA damage repair inhibitors can sensitize cancer cells with the main goal to maximize the cytotoxic effect of therapy [10]. Despite disease-free survival improvement, which was achieved by combination therapy, conventional therapy is still accompanied by the significant collateral damage of non-malignant tissues. To achieve better efficiency of conventionally used drugs, we have focused our research on natural compounds with the main aim to promote better efficiency of 5FU therapy leading to a better tolerated treatment.

For many centuries, natural compounds have been used mainly in Eastern medicine. However, many of currently used chemotherapeutics have even their origin in nature, for instance vincristine, irinotecan, etoposide and paclitaxel are plant-derived compounds. Actinomycin D, mitomycin C, bleomycin, doxorubicin and l-asparaginase are drugs derived from microbial sources, and cytarabine is the first drug originating from a marine source [11]. Natural compounds can target multiple signaling pathways in the cell or organism such as apoptotic and cell cycle pathways [12]. *Ganoderma Lucidum* (GLC), also known as the mushroom of longevity, is a natural compound used in traditional Chinese medicine for more than two thousand years [13]. GLC contains a number of biologically active components, such as triterpenes and polysaccharides [14]. Currently, GLC has been extensively studied from prevention and therapy point of view in many human disorders including cancer (for rev. see [15–17].

We propose that the modulation of DNA damage by natural compounds, particularly GLC, may lead to sensitization of cancer cells to conventional chemotherapeutics and to selective cancer cell death. Potentiation of anticancer effects of conventional chemotherapeutic drugs by well tolerated natural compounds may reduce the effective curative dose of drugs, modify their side effects and lead to better quality of life of CRC patients. Therefore, we have focused on the effect of the GLC on proliferation, migration, cell cycle progression and DNA damage in CRC cell lines as well as in a non–malignant colorectal cell line. To prove our hypothesis, we evaluated the effect of GLC on 5FU treatment both *in vitro* and *in vivo*.

2. Material and methods

2.1. Ganoderma Lucidum (GLC)

GLC was obtained from Pharmanex (Provo, UT, USA, batch No.: DL12561, Shanghai R&D, Pharmanex). GLC had well defined formulation; it contained 6% of triterpenes, 13.5% of polysaccharides. GLC was dissolved in dimethyl sulfoxide (DMSO, Sigma Aldrich, St. Louis, MO, USA) at the concentration of 50 mg/ml and stored at 4 °C.

2.2. Cell treatments

2.2.1. GLC treatment

Fifty mg/ml stock solution of GLC was dissolved in culturing medium to final concentrations 0.25 and 0.5 mg/ml. Medium without GLC was used as a control. Used concentrations were chosen according to results published by Jiang and Sliva [18].

2.2.2. 5FU + GLC co-treatment

5-Fluorouracil (5FU, Sigma Aldrich, St. Louis, MO, USA) was dissolved in DMSO to 500 mM stock solution. For simultaneous co-treatment (5FU + GLC) cells were treated with 5μM 5FU and 0.5 mg/ml GLC. Medium without GLC was used as a control.

2.3. Cell cultures

Human adherent colorectal cancer cell lines HCT116, HT29, HCT116$^{p53-/-}$ were a kind gift from Dr. Andera, Biotechnology and Biomedicine Centre of the Academy of Sciences and Charles University in Vestec (Prague, Czech Republic); originally obtained from ATCC (Manassas, USA). Cells were cultured in DMEM medium (Sigma Aldrich, St. Louis, MO, USA) with 10% fetal bovine serum (Sigma Aldrich, St. Louis, MO, USA), 1 mM L-glutamine (Biosera, Nuaille, France), 1 mM sodium pyruvate (Biosera, Nuaille, France) and 1 mM penicillin/streptomycin (Biosera, Nuaille, France). Non-cancer human colon mucosal epithelial cell line (adherent) NCM460 cells (originally obtained from INCELL Corporation, San Antonio, TX, USA by Prof. Sliva) were cultured in M3:10™ medium (INCELL, San Antonio, TX, USA) with 10% fetal bovine serum (Sigma Aldrich, St. Louis, MO, USA) and 1 mM penicillin/streptomycin (Biosera, Nuaille, France). All cells were cultured in a humidified incubator at 37 °C, 5% CO_2. Cells were used up to 8 passage.

CT26.WT mouse adherent colon cancer cell line was a kind gift from Prof. B. Rihova, Institute of Microbiology of the Czech Academy of Sciences (Prague, Czech Republic), originally obtained from ATCC (Manassas, VA, USA). Cells were cultured in RPMI 1640 medium (Sigma Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Sigma Aldrich, St. Louis, MO, USA) and 1% penicillin/streptomycin solution (Biosera, Nuaille, France) at 37 °C in 5% CO_2 incubator.

2.4. Colony forming assay

Cells were plated on 6 well plates (500 cells/well) and treated with different concentrations of GLC (0.25-0.5 mg/ml) for 24 h. Each tested concentration, as well as control, were performed in triplicates. After 24 h, the medium was replaced with fresh medium. After 12 days, colonies were fixed with 3% formaldehyde and stained with 1% crystal violet. Percentages of colonies were measured in ImageJ software [19].

2.5. Cell proliferation assay

To measure the proliferation; cells were seeded on 96 well plates (5×10^4 cells per well) and treated with GLC (0.25-0.5 mg/ml) in quadruplicates, at different time points (24–72 h). WST1 cell proliferation assay (Roche, Basel, Switzerland) was used according to the manufacturer's protocol (10 μl WST1 reagent per 100 μl of medium, 40 min incubation time). Absorbance was measured using fluorescence reader Biotek ELx808 (Biotek, Vermont, USA), Ex/Em 450/690 nm.

2.6. Migration assay

Cells were seeded to 6 well plates (5×10^5 cells/ml) and treated with GLC extract (0.5 mg/ml) for 24 h. Cell migration was assayed using Transwell Permeable Supports 8.0 μm (Corning, Sigma Aldrich, St. Louis, MO, USA). Cells were seeded in a density of 1×10^4 on the top of a transwell support in 24 well plate format and cultured in DMEM medium supplemented with 0.5% FBS. Cells were allowed to migrate for 24 h through the membrane into the lower part of chamber containing DMEM with 20% FBS. The migrated cells were fixed with 3% formaldehyde, stained with 1% crystal violet and counted in four random fields under 200 x magnification.

2.7. Reactive oxygen species (ROS) measurement

Cells were cultured in 24 well plates (5×10^5 cells/ml). For GLC treatment; cell lines were treated with 0.25 and 0.5 mg/ml concentrations of GLC for 3, 6, 24 h. After incubation, cells were harvested by trypsinization, washed with PBS, and centrifuged (1000 rpm, 10 min). 1 µl of cell-permeant 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) (10 µM, Thermo Fisher) was added to cell pellet and incubated for 30 min at 37 °C. The level of relative fluorescence was measured on fluorescent reader Biotek (Vermont, VT, USA) at Ex/Em: 485/538 nm. For the ROS measurement after the 5FU + GLC co-treatment; cells were treated simultaneously with 0.5 mg/ml GLC and 5µM 5FU and then processed in the same way as described above.

2.8. Measurement of SBs and oxidative DNA damage using comet assay

DNA damage measurements were performed by alkaline comet assay modified for digestion of nucleoids with DNA repair endonucleases (or single cell gel electrophoresis), fully described in Azqueta et al. [20]. Cells were treated with GLC solutions for 90 min, non-treated cells were used as control. Investigated cells were embedded in duplicates in agarose (2×10^5 cells/ml, 0.5% low melting point agarose in PBS, 37 °C) on a microscope slide that was pre-coated with 1% normal melting point agarose dissolved in distilled water. The slides were then immersed in cold lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Trizma Base, 1% Triton X-100, pH = 10, 4 °C) for 1 h in order to obtain substrate DNA in the form of nucleoids fixed in agarose. Subsequently, slides were washed in washing buffer (40 mM HEPES, 0.5 mM EDTA, 0.2 mg/ml BSA, 0.1 M KCl, pH = 8, 3 changes, 5 min each at 4 °C).

Regarding detection of specific oxidative DNA damage, half batch of the nucleoids was incubated with the formamidopyrimidine DNA glycosylase enzyme (Fpg, New England Biolabs, Ipswich, MA, USA), dissolved in reaction buffer (40 mM HEPES, 0.5 mM EDTA, 0.2 mg/ml BSA, 0.1 M KCl, pH = 8, 4 °C) for 30 min at 37 °C. The second part of nucleoids was incubated with reaction buffer only for 30 min at 37 °C to detect SBs. Alkaline incubation followed (freshly prepared - 0.3 M NaOH, 1 mM EDTA, 4 °C) for 30 min in dark, converting alkali-labile sites to SBs. During electrophoresis (1.19 V/cm, 300 mA, 40 min, 4 °C, dark) in the same alkaline buffer, DNA loops containing SBs were drawn towards the anode forming a comet-like image. Slides were then washed in 1xPBS (4 °C) for 10 min, in distilled water (4 °C) for 10 min and dried overnight. Next day, slides were stained with SYBR Gold (Invitrogen, Carlsbad, CA, USA) diluted 1:10.000 in TE buffer. Comets were visualized with fluorescence microscope Olympus BX63 (Olympus, Tokyo, Japan) and analyzed using semi-automated Lucia Comet Assay™ software (Laboratory Imaging, Prague, Czech Republic). One hundred comets were scored per gel (*i.e.* two hundred comets per slide). Median tail intensity (TI), reflecting the frequency of DNA in tail (% tail DNA), per gel and then the mean TI of replicate gels was used as the parameter to describe the comets. The level of specific oxidative DNA damage was expressed as net values (the level of SBs detected on slides incubated with Fpg minus the level of SBs detected on slides incubated with reaction buffer).

2.9. Cell cycle analysis

Cells were seeded on 12 well plates (5×10^5 cells/ml) and treated with 0.5 mg/ml GLC for 12–72 h. After the treatment, cells were harvested by trypsinization, washed with PBS and spun down at 1000 rpm for 10 min. Then, 1 ml of Propidium iodide (PI) staining solution (0.02 mg/ml PI, 0.02 mg/ml RNase, 0.05% Triton X-100) was added to the cell pellet and cells were incubated for 30 min at 37 °C in the dark. After incubation, samples were measured using flow cytometer (Apogee A-50 micro, Apogee, Hertfordshire, UK). Measured data were analyzed with Flowlogic software (Inivai Technologies, Mentone, Australia).

2.10. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis

Cells after treatment were washed with PBS. Sixty µl of TTL buffer (1 M Tris-HCl, 5 M NaCl, 0.2 M EDTA, 10% Triton X, protease inhibitors) were added to each well and cells were harvested by cell scrappers and transferred into tubes. The tubes were frozen on dry ice. After 15 min cells were gently thawed and incubated for 20 min on ice. After incubation cells were spun down at 20 000xg, 20 min, 4 °C. The supernatants were aspirated into new tubes. The concentrations of proteins were measured by Bradford reagent (Sigma Aldrich, St. Louis, MO, USA) according to manufacturer recommendations. Proteins (20 µg) were loaded and separated in 12% SDS-PAGE gels at 15 mA for 60 min. Then, the separated proteins were transferred to 0.45 µm Amersham Protran Nitrocellulose Blotting Membrane (GE Healthcare, Life science) in methanol transfer buffer using Mini Trans-Blot Cell (Bio-Rad Laboratories, CA, USA). The membranes were blocked with 5% BSA in Tris-buffered saline containing Tween 20 (TBST; 20 mM Tris–HCl, pH 7.4, 0.15 M NaCl, and 0.1% Tween 20) for 1 h and incubated with anti-p53 (Cell Signaling, Leiden, The Netherlands) and anti-GAPDH (Abcam, Cambridge, UK) at 4℃ overnight, followed by incubation with goat anti-rabbit secondary antibody conjugated with horseradish peroxidase (Abcam, Cambridge, UK). The membranes were then incubated with SupersignalWest Pico Chemiluminiscent Substrate (Pierce, Thermofisher, Massachusetts, USA) and visualized by Azure c600 (Azure Biosystems, Dublin, CA, USA).

2.11. Mice and tumor induction and treatment

Three-month old female BALB/c mice were purchased from Institute of Physiology of the Czech Academy of Sciences (Prague, Czech Republic). All mice were maintained and handled in accordance with the procedures approved by the Institute of Microbiology animal care and use committee (No. 105/2016). Thirty-two mice were inoculated subcutaneously on the right side of their shaved back with a single-cell suspension of CT26.WT cells (200,000 cells in 100 µl) and, after 10 days, divided into four groups with eight mice each. All groups received single or combined therapy when the tumors reached average volume of about 300 mm^3 (day 14). The mice were gavaged daily with 100 µl suspension of GLC powder in sterile distilled water (110 mg/ml) according Sliva et al. [21] and injected intraperitoneally three times a week with 200 µl 5FU (Sigma Aldrich, St. Louis, MO, USA) solution in sterile phosphate buffered saline (20 mg/kg). The tumor dimensions were measured twice a week by a caliper and tumor volume was calculated using a formula (length x $width^2$)/2. The mice were sacrificed on day 48. Tumor samples dimensions were measured by caliper and weight out. After measurement tumor tissues were frozen.

2.12. Statistical analysis

Statistical analyses were performed using pairwise comparison by Student's *t*-test and Two-way ANOVA (GraphPad Prism5, GraphPad Software, La Jolla California USA, www.graphpad.com). The results represent the mean value of three independent experiments ± SD; the significance level was set at $p \leq 0.05$.

3. Results

3.1. Ganoderma Lucidum inhibits growth and invasive behavior of colorectal cancer cell lines

To define the effect of GLC on cell proliferation, HCT116, HT29 and non-malignant NCM460 cells were treated with two different doses of GLC (0.25 mg/ml and 0.5 mg/ml). After 24 h treatment we observed non-significant decrease in cell proliferation (data not shown). However, we recorded significantly decreased cell proliferation in

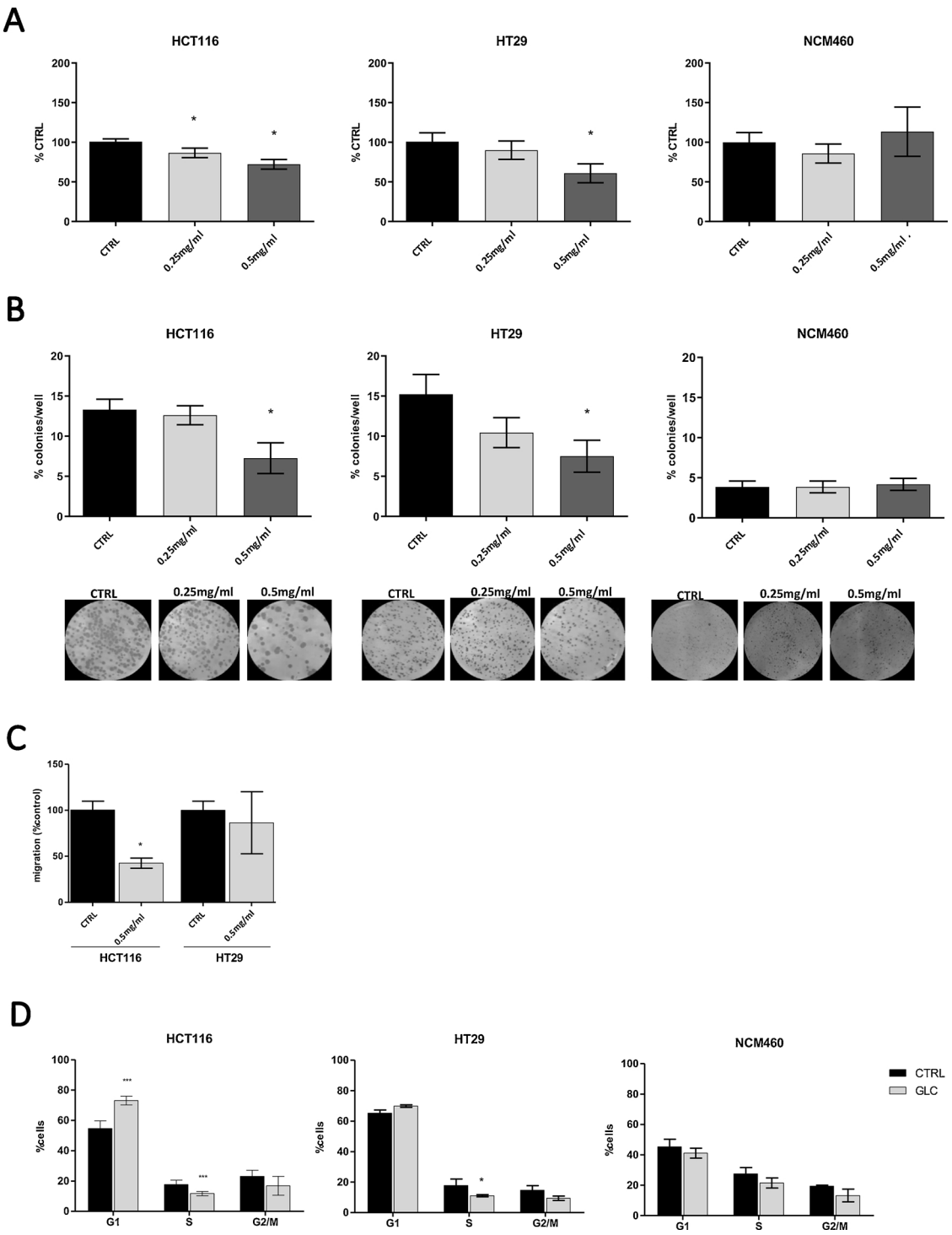

Fig. 1. GLC inhibits growth, invasive behavior and cell cycle of colorectal cancer cell lines.
(A) Cell proliferation measured by WST assay after 48 h GLC treatment in HCT116, HT29 and NCM460 cells. (B) Colony forming assay after GLC treatment. (C) Migration of cancer cells after GLC treatment. (D) Propidium iodide analysis to define cell cycle distribution after GLC treatment. All data are expressed as means ± SD of triplicates and *p ≤ 0.05 *versus* control (non-treated cells).

HCT116 (by 27%, $p < 0.05$) and HT29 (by 39%, $p < 0.05$) cancer cells at 0.5 mg/ml after 48 h treatment. The prolonged GLC treatment for 72 h resulted in persisting lower proliferation in HCT116 cells, whereas no effect on proliferation was recorded in HT29 cells (data not shown). Proliferation of non-malignant cells was not affected (Fig. 1A).

Colony forming assay was performed to verify the anti-proliferative potential of GLC. CRC and non-malignant cells were treated with two different doses of GLC (0.25 mg/ml and 0.5 mg/ml) for 24 h as described in Materials and Methods. After 0.5 mg/ml GLC treatment, the number of colonies significantly decreased by 46% and 45% in HCT116 and HT29 cells, respectively ($p < 0.05$), when compared to non-treated cells (Fig. 1B). GLC treatment did not affect non-malignant NCM460 cells (Fig. 1B). In following experiments, the cells were treated with 0.5 mg/ml GLC, because this concentration showed higher efficacy in our experiments. The effect of GLC on migration of cancer cells was analyzed as well. Significant 57% decrease in migration of HCT116

cells was observed after GLC treatment ($p < 0.05$), however HT29 cells showed only moderate reduction by 14% (Fig. 1C). The cell cycle distribution after GLC treatment was analyzed by flow cytometry after propidium iodide staining. We did not observe any difference in cell cycle distribution in either cell line treated with 0.5 mg/ml GLC for 24 h (data not shown). Fig. 1D is a representative figure depicting effects on cell cycle distribution after 48 h treatment with 0.5 mg/ml GLC. At this time interval significant increase in the amount of HCT116 cells in G1 phase and decrease in the amount of cells in S phase ($p < 0.001$) was pronounced. The same tendency was observed in HT29 cells as well; the reduced proportion of cells in S phase was significant ($p < 0.05$). This indicates the GLC induced G1/S cell cycle arrest. Moreover, in HT29 cells, several cells in G0 phase were observed.

3.2. The effect of Ganoderma lucidum treatment on oxidative DNA damage

To asses DNA damage by GLC, the CRC cells were treated with 0.5 mg/ml GLC extract for 90 min. GLC treatment induces significant changes in the amount of DNA strand breaks in HCT116 cell line ($p < 0.05$) but not in HT29. However, the levels of oxidative DNA damage significantly increased in HCT116 and in HT29 cells, respectively (both $p < 0.05$, Fig. 2A and 2B). We found no increase in DNA strand breaks and oxidative DNA damage in non-malignant reference cells – NCM460 (Fig. 2C).

3.3. GLC enhances the effect of 5FU in CRC cells

Our results showed that GLC specifically decreased colorectal cancer cell growth and also induced DNA damage. In the next part of our study, we tested the efficacy of GLC in combined treatment with conventionally used chemotherapeutic 5FU.

Cancer cells simultaneously treated with GLC (0.5 mg/ml) and 5FU (5 μM) were analyzed for proliferation, long term survival (colony formation), and DNA damage.

GLC co-treatment with 5FU did not significantly affect cancer cell proliferation in HCT116 (Fig. 3A). However, growth of HCT116 cells was decreased by about 20% compared to the effect of 5FU alone ($p < 0.01$, Fig. 3B). In HT29 cells, GLC enhanced the effect of 5FU by about 15% ($p < 0.05$, Fig. 3B). For further validation, the co-treatment effect was analyzed using Combenefit software [22]. This analysis showed an additive effect of GLC and 5FU treatment (data not shown).

The co-treatment (GLC + 5FU) also increased the level of DNA strand breaks in HT29 cells and oxidative damage in HCT116 cells (Fig. 3C). In HCT116 cells, simultaneous treatment with GLC and 5FU increased the level of oxidative DNA damage ($p < 0.05$) in comparison with 5FU treatment alone. In HT29 cells, co-treatment increased DNA strand breaks ($p = 0.05$). We did not observe any effect of GLC as well as simultaneous treatment of 5FU + GLC on any of the analyzed parameters in non-malignant colonic NCM460 cells (summarized in Fig. 3).

3.4. Effect of Ganoderma lucidum on accumulation of reactive oxygen species (ROS) in CRC and non-malignant cell lines

The effect of GLC treatment (0.25 mg/ml and 0.5 mg/ml) on ROS production in CRC and non-malignant cell lines was examined after 3, 6, 24 h incubation. We did not detect any changes in ROS levels after the 3 h treatment (data not shown), whereas 6 h treatment with 0.25 mg/ml GLC induced an increase in ROS levels in HCT116 cells, treatment with 0.5 mg/ml was non significantly increased (Fig. 4A, $p < 0.05$). In HT29 cells, we detected non-significant increase in ROS accumulation. In non-malignant NCM460 cells, the treatment with both doses of GLC extract caused a significant decrease in ROS levels by about 20% after 6 h (0.25 mg/ml, $p < 0.05$; 0.5 mg/ml, $p < 0.01$) and by about 17% after 24 h (0. 25 mg/ml, $p < 0.05$; 0.5 mg/ml, $p < 0.001$, Fig. 4A).

3.5. Role of p53 in GLC treatment

The effect of GLC treatment on level of p53 protein was analyzed in all tested cell lines. GLC (0.5 mg/ml) treatment increased the level of p53 in CRC cell lines (Fig. 5A). In NCM460, the level of p53 was not changed in comparison with non-treated control. HCT116$^{p53-/-}$ cells were used to investigate the role of p53 protein in GLC effect. HCT116$^{p53-/-}$ cells were treated with different concentrations of GLC for 48 h. Our results showed a 42% decrease in cell proliferation after treatment with 0.5 mg/ml GLC ($p < 0.05$, Fig. 5B). GLC co-treatment with 5FU also decreased the cell proliferation by 17% ($p < 0.05$, Fig. 5B). This effect was also confirmed by colony forming assay showing significant decrease in cancer cell growth (Fig. 5C). We did not observe any effect on ROS accumulation in HCT116$^{p53-/-}$ cells (Fig. 5D). GLC treatment induced DNA strand breaks and oxidative DNA damage. GLC co-treatment with 5FU enhanced the effect of 5FU on both types of DNA damage ($p < 0.05$, Fig. 5E).

3.6. Effect of GLC on 5FU in mice xenograft model in vivo

To confirm the effect of GLC on 5FU treatment *in vivo*, we used mice transplanted with syngeneic CT26 cells. After 14 days of tumor growth, we started to treat the mice with GLC alone or in combination with 5FU. Although non-significant, in GLC + 5FU group, we observed better survival ($p = 0.0628$) and smaller tumor volume in comparison to other groups (Fig. 6A and 6B). These findings were also associated with lower tumor weight measured at the day of experiment termination ($p < 0.05$, Fig. 6C).

4. Discussion

Ganoderma Lucidum (family *Ganodermataceae)* is basidiomycetous fungi used in traditional Eastern medicine for centuries. This medical mushroom is believed to preserve human vitality and promote longevity. It has been used to treat various human diseases, such as allergy, arthritis, bronchitis, gastric ulcer, hyperglycemia, hypertension, chronic hepatitis, hepatopathy, insomnia, nephritis, neurasthenia, scleroderma, inflammation, and cancer [23]. In our study, we have

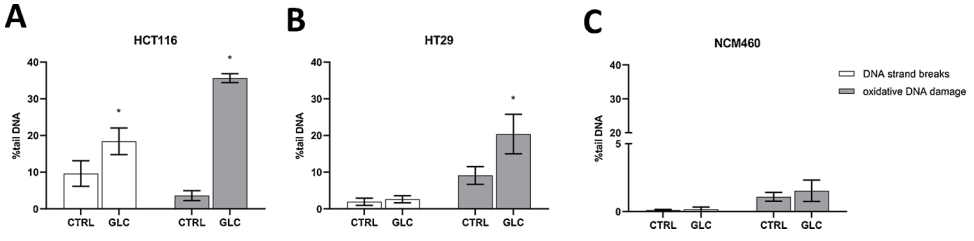

Fig. 2. Effect of GLC on DNA damage.
(A) Effect of GLC on DNA strand breaks and oxidative DNA damage measured by comet assay in HCT116. (B) Effect of GLC on DNA strand breaks and oxidative DNA damage in HT29. (C) Effect of GLC on DNA strand breaks and oxidative DNA damage measured in NCM460. Level of oxidative DNA damage is expressed as net values. All data are expressed as means ± SD of triplicates and *$p \leq 0.05$ *versus* control (non-treated cells).

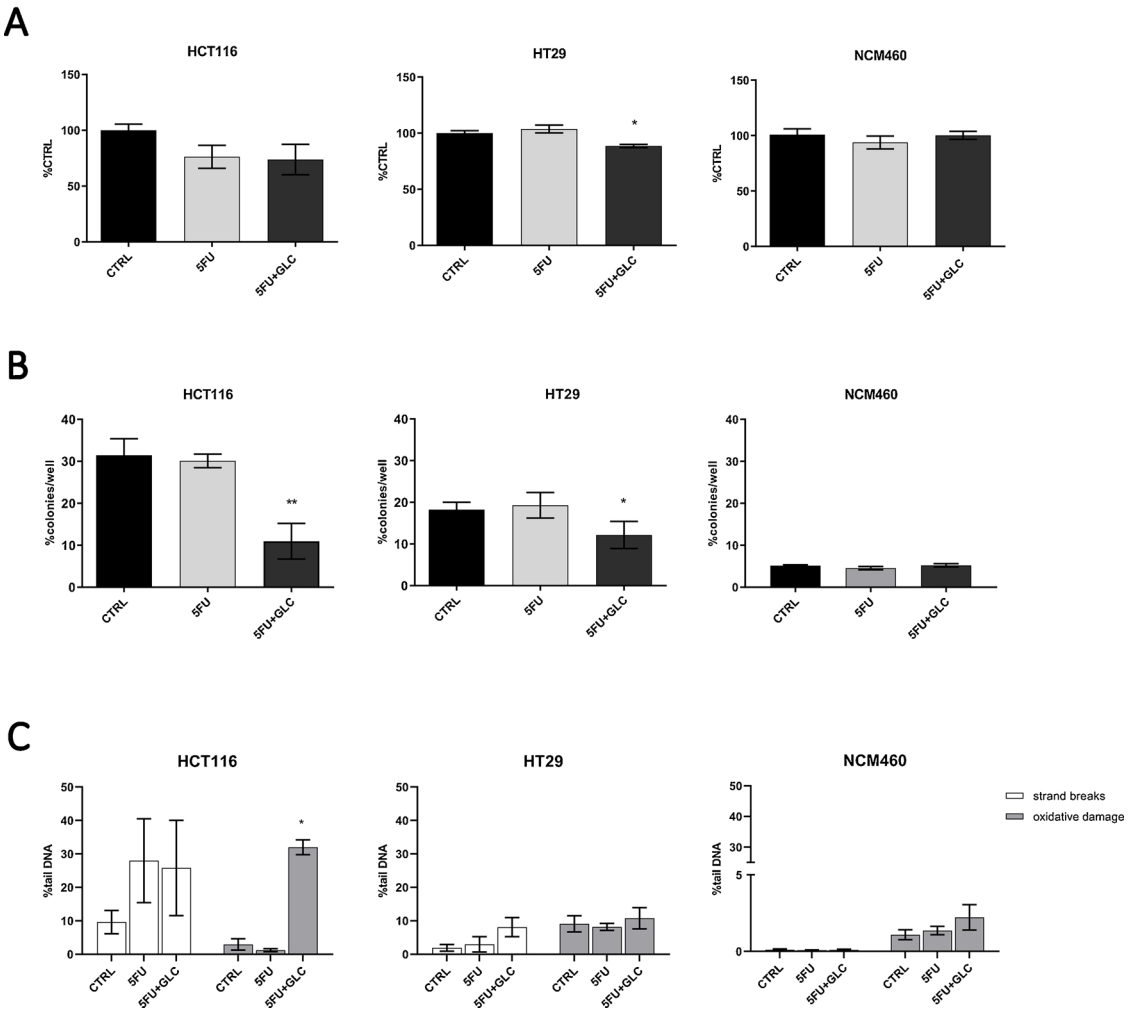

Fig. 3. The effect of GLC in combination with 5FU *in vitro*.
(A) Proliferation after GLC and 5FU co-treatment. (B) Effect of GLC and 5FU co-treatment on colony forming assay. (C) Effect of GLC and 5FU co-treatment on DNA damage in HCT116 and HT29 CRC cell lines. All data are expressed as means ± SD of triplicates and *p ≤ 0.05 *versus* control (non-treated cells).

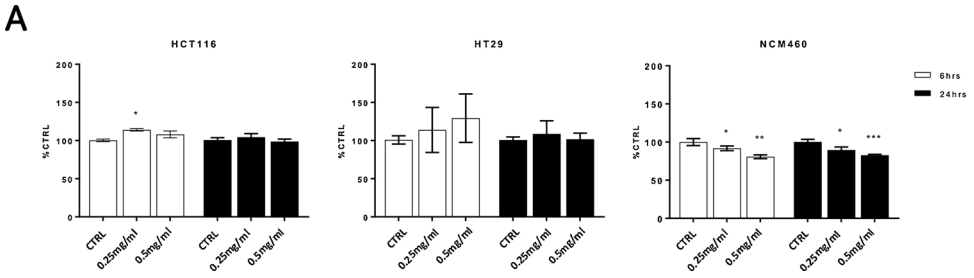

Fig. 4. GLC effect on ROS production in colorectal cell.
(A) Intracellular ROS accumulation after GLC treatment detected by 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) in HCT116, HT29 and NCM460 cells after 6 h and 24 h GLC treatment. All data are expressed as means ± SD of triplicates and *p ≤ 0.05 *versus* control (non-treated cells).

tested the consequence of simultaneous treatment of natural compound GLC together with chemotherapeutic 5FU on colorectal cancer, both *in vitro* and *in vivo*. In our recent review article we hypothesized that potentiating of anti-cancer effects of chemotherapeutics with well tolerated natural compounds may modify the effective drugs dose, diminish their side effects and ultimately lead to a better quality of life for cancer patients [17].

Our original hypothesis that co-treatment of GLC with 5FU enhances its cytotoxic effect in CRC was confirmed in our study. Simultaneous treatment of CRC cells with GLC and 5FU led to increased level of oxidative DNA damage resulting in significantly decreased cancer cell growth. Similar effect on colorectal cancer cells was shown by Jiang et al., who showed that *Ganoderma Lucidum* polysaccharides (GLPs) administered in combination with 5-FU synergistically suppress proliferation of CRC cells [24].

These data suggest that specific DNA damage caused by natural compounds may become a potential tool for improvement of the anticancer treatment. Furthermore, we investigated the effect of GLC on cancer cell proliferation, migration, cell cycle progression, as well as DNA damage in malignant CRC cells and non-malignant colorectal cell line. Additionally, we have tested a well characterized GLC extract containing both polysaccharides and triterpenes and observed that GLC decreased proliferation of HCT116 and HT29 cancer cells. Recently, many authors brought an evidence, that GLC, particularly its component triterpenes, decreases cancer cell proliferation in ovarian [25], breast [26,27] and also in colorectal cancer cells [28,29]. Moreover, to our best knowledge, we have documented for the first time that GLC has no significant effect on non-malignant colorectal cells. To further

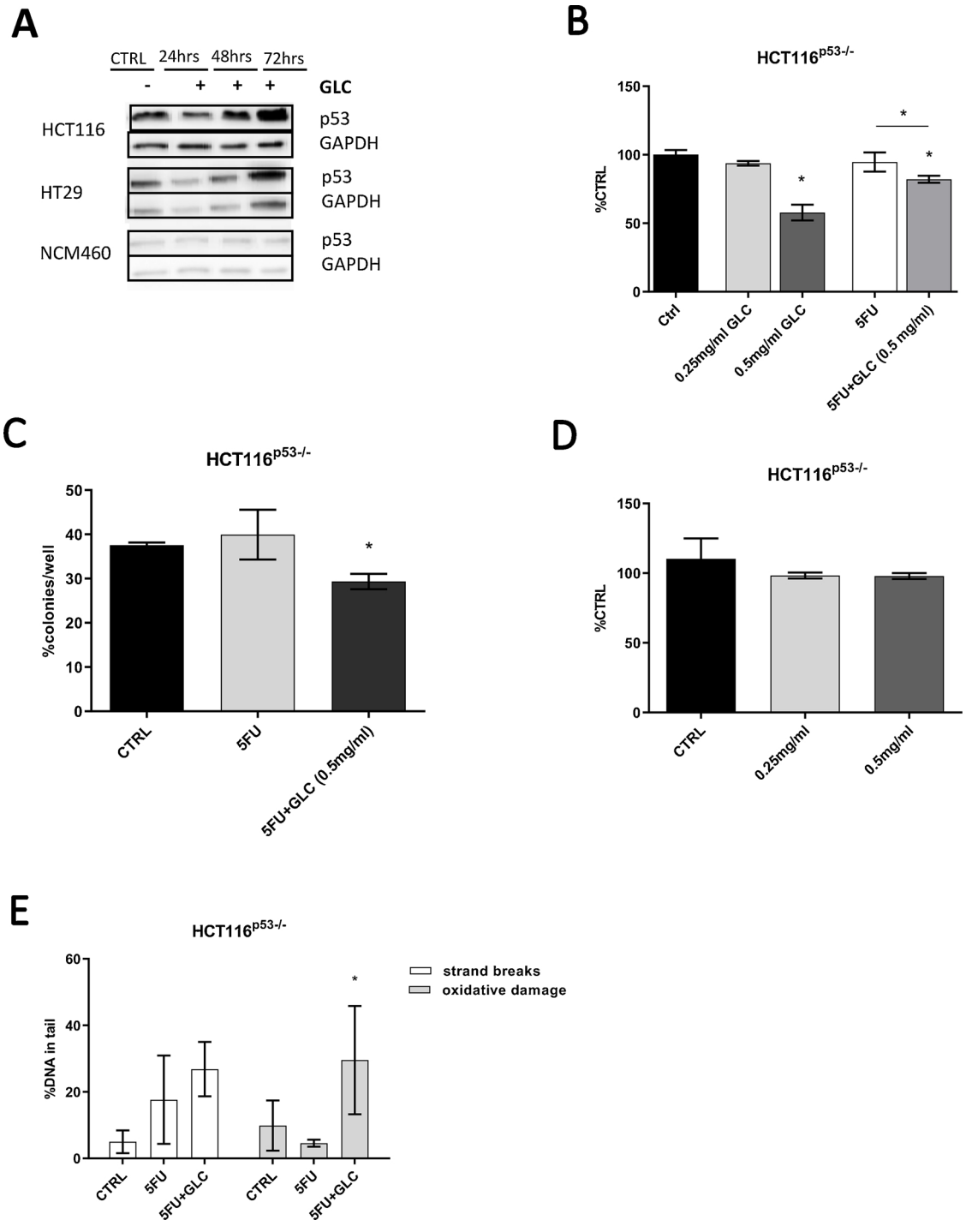

Fig. 5. The role of p53 in GLC treatment.
(A) Representative figure of western blot analysis of p53 level after GLC treatment. (B) Proliferation of HCT116$^{p53-/-}$ cells after GLC treatment alone and in 5FU co-treatment. (C) Effect of co-treatment of 5FU with GLC on colony forming assay. (D) ROS accumulation after GLC treatment. (E) Level of DNA damage after 5FU and GLC co-treatment. All data are expressed as means ± SD of triplicates and *p ≤ 0.05 *versus* control (non-treated cells).

investigate the anti-cancer effect of GLC, we also analyzed cells long term survival, which defines the ability of single cell to divide [30]. We detected decreased growth of colorectal cancer cells after GLC treatment, while the growth of non-malignant colorectal cells NCM460 remained unaffected. Another important feature of tumor cells is their invasiveness. Cell migration is a crucial process for normal development and homeostasis, but disturbed cellular migration is also an essential trait for cancer metastasis development. This metastatic spread of the primary tumor accounts for over 90% of patient`s mortality associated with solid tumors [31]. Subsequently, we have observed that GLC significantly reduced also cancer cell migration. Consistently with our results, Li et al. reported that ethanol extract of Ganoderma triterpenes suppressed HCT116 migration through the upregulation of E-cadherin [32]. This effect was also described in breast cancer cells by Martínez-Montemayor et al. [33]. It is well recognized that the inhibition of cancer cells growth and their invasive behavior are important mechanisms for carcinogenesis inhibition. In accordance with this assumption, we proved that GLC showed a pronounced antitumor effect

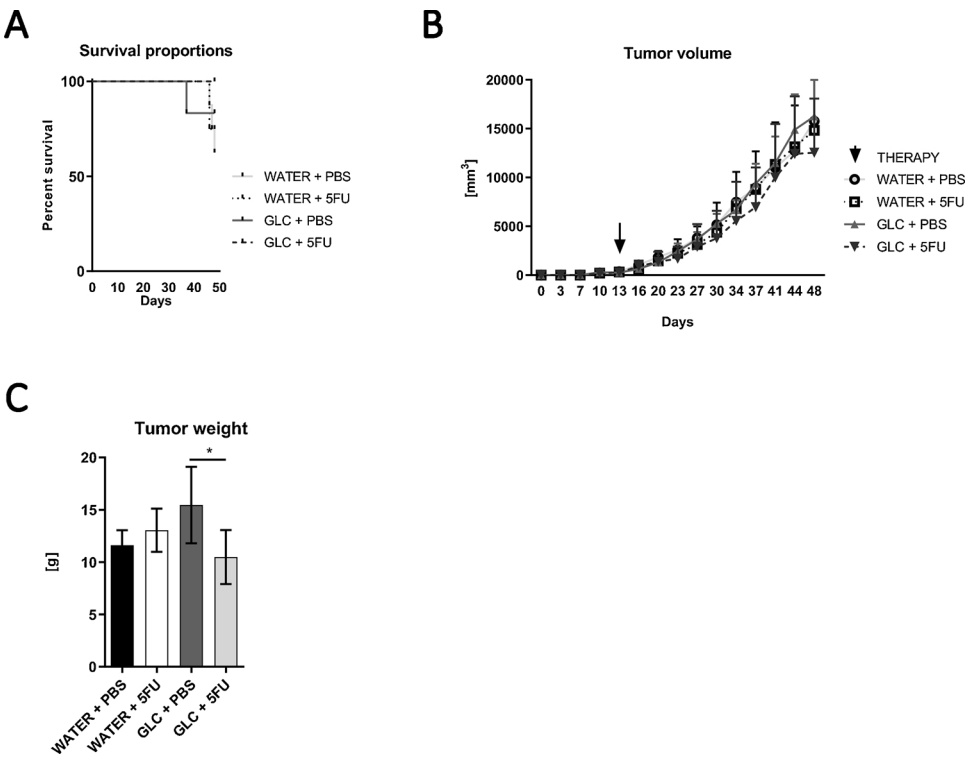

Fig. 6. The effect of GLC and 5FU *in vivo*. (A) Kaplan Meier curves representing mice survival after GLC and 5FU administration. (B) Differences in tumor volume after GLC and 5FU administration. (C) Differences in tumor weight after GLC and 5FU administration. All data are expressed as means ± SD and *$p \leq 0.05$ *versus* control group.

on CRC cells without affecting non-malignant cells. This may suggest protective effect of natural compounds on non-malignant cells and explain why the natural compounds are well tolerated in humans.

To validate our results from *in vitro* studies, we analyzed the simultaneous effect of GLC with 5FU in a mice xenograft model. The group of mice treated with GLC and 5FU together exhibited better survival. GLC also positively influenced the cytotoxic effect of 5FU on tumor size. Overall fitness of animals is important in evaluation of the toxicity and the side effects of a chemotherapy drug or a natural supplement in animal studies. Groups treated with GLC showed a moderate increase in body weight (data not shown) and had better overall fitness than mice in other groups. Sliva et al. reported that GLC triterpenes could be used as an alternative dietary approach for the prevention of cancer associated colitis [21] and Xu et al. showed that GLC attenuated doxycycline induced cardiotoxicity [34]. Zhao el al described that GLC enhances the sensitivity of ovarian cancer cells to cisplatin [35], and Yue et al. defined synergism between GLC triterpenes and doxorubicine [36]. Li et al. showed that *Ganoderma microsporum* prevented 5FU induced mucositis in mice model [37]. However, studies addressing effect of GLC on 5FU treatment in mice tumor xenograft model are rather scarce. Taking together, we proved that GLC could enhance cytotoxic effect of 5FU, and alongside it protects non-malignant cells from 5FU cytotoxicity.

In our study, we observed important effect of GLC and 5FU on CRC and non-malignant cells. To further understand this phenomenon, we focused on the description of the mechanism involved in the GLC cytotoxicity. Since DNA damage is a complex target for anticancer drugs [38], we focused on the level of DNA strand breaks after GLC treatment. We detected an accumulation of DNA strand breaks (HCT116) in CRC cells after GLC treatment. Furthermore, we documented that GLC induced specific oxidative DNA damage in CRC cells. Some studies reported that triterpenoids isolated from GLC induced oxidative DNA damage due to their structure-activity relationships. Liu et al. suggested that oxidative DNA damage accumulation depends on the degree of acetylation in the structure of GLC triterpenoid [39]. It is well known that many natural compounds are able to cause oxidative DNA damage, such as curcumin [40], resveratrol [41] and *Ginkgo Biloba* extract [42] ultimately resulting in cell cycle arrest and apoptosis of colorectal cancer cells [43]. The importance of studying natural compounds in the cancer therapy is supported by the fact that some of these natural compounds recently underwent clinical trials (curcumin), [44,17].

Under physiological conditions, recognition of DNA damage induces the DNA damage response (DDR) machinery in order to maintain genomic integrity of the cell. Suboptimal activity of DDR may enhance the effect of DNA damaging compounds. Kuo et al. reported that dietary flavonoids can enhance chemotherapeutic effect by inhibiting DDR [46]. We demonstrate that, GLC increased the accumulation of oxidative DNA damage. On the contrary, GLC treatment significantly decreased the level of ROS in non-malignant colorectal cells. Reactive oxygen species can react with different components of DNA and cause DNA lesions. These properties predestine ROS as a potential target for anti-cancer therapy [47]. There is an evidence of protective effect of GLC against ROS formation, mostly in non-cancerous cells. Li et al. has already postulated that GLC polysaccharides exert a protective effect against oxidative stress in the brain cells [48] as well as in cardiomyocytes [34].

Our results showed that HT29 are less sensitive to GLC treatment. The reason for this difference could be due to the fact that HT29 line bears a mutation in *TP53* gene [49]. Mutation in *TP53* is often present in many cancer types, including advanced CRC. Loss of p53 function by mutations leads to uncontrolled cell cycle progression [50]. Jiang et al. published that polysaccharides from GLC restore tumor suppressor function of mutant p53 [24]. Moreover, we observed increased levels of p53 protein after GLC treatment. We hypothesized that restoration of tumor suppressor function of p53 after GLC treatment may lead to restoration of cell cycle regulation and cell cycle arrest and apoptosis. To define the role of p53 protein in GLC treatment, we used HCT116$^{p53-/-}$ cells. p53 in general is not necessary for the induction of the DNA damage, but it is critical for the occurrence of the cell death. In the presence of p53, GLC dramatically enhanced cytotoxicity of 5FU by triggering of the oxidative DNA damage. In case of p53$^{-/-}$ cells, GLC induced the DNA damage, and simultaneously reactivated p53. These changes may subsequently result in cell growth inhibition and apoptosis, as recently shown by Jiang et al. [24].

5. Conclusion

In summary, GLC showed a substantial effect on CRC cells by induction of oxidative DNA damage, whereas it protected non-malignant cells from ROS accumulation. Moreover, GLC enhanced the toxic effect of 5FU in CRC cell lines. According to obtained results, we propose natural compounds may represent a promising supplement to conventional cancer therapy, which may finally reduce the effective curative dose of anticancer drugs and improving patients' outcomes.

Conflict of interests

The authors declare that there are no conflicts of interest.

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