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Antiproliferative Activity of Melanoidins Isolated from Heated Potato Fiber (Potex) in Glioma Cell Culture Model

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ABSTRACT: Potex constitutes a potato fiber preparation widely used as an ingredient to meat and bakery products which thermal treatment results in creation of new compounds. Melanoidins are high molecular weight brown end products of Maillard reaction, and few data presenting tumor cell growth inhibiting activity of melanoidins have been reported. Thus, in present study we utilized water extract of Potex roasted (180 °C for 2 h), whose chemical characterization revealed the presence of melanoidin complexes. Heated Potex extract inhibited C6 glioma cell proliferation in a dose-dependent manner measured by MTT method. High molecular weight components present in initial extract were responsible for stronger antiproliferative effect compared with low molecular weight fraction. Impaired MAPK (mitogen-activated protein kinase) and Akt signaling was found in cells treated with the extract. Moreover, flow cytometry analyses revealed the extract to induce G_1/S arrest in glioma cells. Simultaneously, Western blot analysis showed elevated levels of p21 protein with concomitant decrease of cyclin D1. In conclusion, observed antiproliferative activity of melanoidins present in heated Potex was linked to disregulated MAPK and Akt signaling pathways, as well as to cell cycle cessation. These results suggest potential application of Potex preparation as a functional food ingredient and chemopreventive agent.

KEYWORDS: antiproliferative effect, cell cycle, melanoidins, potato fiber, protein kinase signaling

INTRODUCTION

Gliomas are prevalent primary brain tumors, and only in United States, around 16000 people are diagnosed every year. Glioblastomas (tumors of astrocytic cell origin, WHO grade IV) constitute the most commonly occurring gliomas and are often associated with very poor survival. It is usually due to their malignancy, invasiveness, and strong migrating potential into adjacent brain tissue resulting in secondary foci creation. Widely used resection, radiotherapy, and chemotherapy are often ineffective, and patients pass away within a year.[1−3] Several signaling pathways, including MAPK (mitogen-activated protein kinase) and Akt, have been identified to be upregulated in malignant gliomas and are considered as key molecular targets for glioma prevention and treatment.[4]

Regardless of enormous difficulties in cancer therapy there is an overall agreement nowadays that it is preferable to prevent the disease than cure its end stage. In accordance with WHO reports, more than one-third of all cancer cases could be prevented. In this field chemoprevention is gaining a lot of attention, and chemopreventive agents are frequently vegetable and fruit derivatives. These usually play also as a chemotherapeutic, exerting the activity to inhibit tumor cell proliferation and inducing apoptosis.[5] A number of diet-derived compounds exert protective effect against glioma development, including tea polyphenol chlorogenic acid,[6] γ-linolenic acid,[7] curcumin,[8] or resveratrol.[9]

In the field of cancer chemoprevention the protective role of dietary fiber is still being questioned.[10,11] Despite discrepancy among findings, a number of studies revealed a strong inverse association between fiber consumption and colon adenomas or cancer.[12,13] Currently, dietary fiber is becoming a highly widespread ingredient of our daily diet. Commercially available potato fibers are being used in meat processing industry[14,15] or bakery[16] to improve quality and organoleptic properties of food. Potex is a potato fiber preparation where dietary fiber constitutes up to 70%, and other ingredients are starch (12%), protein (5%), ash (4%), water (9%), and negligible amount of fat (0.3%). Due to its high water retention capacity and ability to absorb oil Potex is broadly applied as an ingredient for meat products, i.e., sausages. It is also utilized as a fat replacer improving texture and stability of these foodstuffs (www.culinar.se).

Thermal treatment of foods is a common treatment before consumption. During heat processing (cooking, baking, and roasting) of foods Maillard reaction takes place and leads to specific color and aroma formation. Resultant color changes are mainly due to melanoidin development. Melanoidins are usually described as brown, high molecular weight final products of Maillard reaction. They are formed as a result of multistage reaction between reducing sugars and compounds possessing free amino groups.[17,18] Heat processing of foodstuffs containing

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Potex is very common, and this may lead to the formation of new compounds from the potato fiber preparation. Due to high amount of polysaccharides and protein, we presume that, among others, melanoidins are formed. Thus, in this study we isolated and fractionated water-soluble melanoidins resultant from heated potato fiber Potex to investigate its activity on the growth of glioma cells in vitro. Preliminary obtained results concerning cancer cell growth inhibiting properties of crude heated Potex extract encouraged us to continue this promising study.[19]

MATERIALS AND METHODS

Cell Culture, Media, Antibodies, and Reagents. C6 rat glioma cells were obtained from the European Collection of Cell Cultures (ECACC, Salisbury, United Kingdom) and maintained in DMEM supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin (Sigma, St. Louis, MO, USA).

For Western blot analysis cells were exposed to control medium alone or with 1000 μg/mL water extract of heated potato fiber Potex for indicated length of time. To examine concentration-dependent effect, cells were treated with various concentrations of the extract (100, 250, 500, and 1000 μg/mL) for 1 h. To compare the activity of different fractions obtained from initial extract, cells were incubated with high (HMW) and low (LMW) molecular weight fractions (1000 μg/mL) for 1 h. For flow cytometry analyses C6 cells were grown in the presence of culture medium or heated Potex extract (500 and 1000 μg/mL) for 24 or 48 h.

Antibodies against phospho-MEK1/2 ($Ser^{217/221}$, 1:1000), phospho-ERK1/2 (Thr^{202}/Tyr^{204}, 1:2000), phospho-p38 (Thr^{180}/Tyr^{182}, 1:1000), phospho- SAPK/JNK (Thr^{183}/Tyr^{185}, 1:1000), phospho-Akt (Ser^{473}, 1:1000), Akt-pan (1:1000), phospho-GSK-3β, cyclin D1 (1:2000), and β-actin (1:2000) were obtained from Cell Signaling Technology, Beverly, MA, USA. Anti-p21 (1:1000) and anti-ERK2 (1:1000) were from Santa Cruz Biotechnology, Santa Cruz, CA, USA. Horseradish peroxidase (HRP) conjugated secondary antibodies were obtained from Cell Signaling Technology. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and rhodamine-conjugated phalloidin (RHPH) were purchased from Sigma. Hoechst 33342 was obtained from Invitrogen, Eugene, OR, USA. Potato fiber (Potex) was obtained from Culinar, Sweden.

Potex Roasting, Melanoidin Isolation, and Purification. For the roasting procedure approximately 25 g of raw Potex was weighed and roasted in a laboratory oven at temperature of 180 °C for 120 min. For these roasting conditions an average 18.5% loss (as is) was obtained, and the Potex fiber acquired a deep brown color. The resulting roasted Potex was extracted 1 h at room temperature with stirring, with 300 mL of water, and the resulting dispersion was filtered under vacuum through a glass fiber filter. The resulting Potex infusion was freeze-dried. The solids obtained represented 9.2% of the roasted Potex. The solids were suspended in 50 mL of water, and the solution was fractionated into a high and a low molecular weight fraction, through ultrafiltration (Amicom, 10 kDa cutoff membrane) with six washings with 200 mL of water. The ultrafiltrate (LMW Potex) and retentate (HMW Potex) were frozen and freeze-dried. The HMW Potex represented 42.7% of the Potex infusion solids and the LMW Potex 56.1%.

HMW Potex fraction was subjected to a second purification procedure by size exclusion chromatography with Sephacryl S-200 HR. The stationary phase was packed on a XK 16/70 with a bed height of 58 cm. The eluent was 100 mM sodium phosphate buffer, pH 6.5, with a flow rate of 1.0 mL/min. The injection volume was 10 mL. The eluent was continuously monitored by refractive index and absorbance at 420 nm. Fractions of 2 mL were collected, and the total sugars were determined colorimetrically by the phenol−sulfuric acid method.[20] The exclusion and total volume of the column was determined with blue dextran and glucose, respectively. The appropriate fractions (HMWF) were collected and dialyzed (12−14 kDa cutoff membrane, Visking size 8; Medicell International Ltd., London, U.K.) at 4 °C with five water renewals. The retentate was frozen and freeze-dried.

Cell Viability Assay. The effect of water extract of heated potato fiber Potex on cell viability was determined with use of MTT assay. Yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was metabolized by viable, metabolically active cells to blue formazan crystals. Measured optical density of resultant colored solution was directly proportional to the number of living cells. C6 cells were seeded onto 96-well microplates at a density of 0.5×10^3cells/well. The next day the medium was replaced with fresh one, alone or with various concentrations of the extract (10, 50, 100, 250, 500, 1000 μg/mL) and incubated for 96 h in standard conditions (5% CO_2, 37 °C). After incubation period, MTT solution (5 mg/mL in PBS) was added for 3 h. Resultant formazan crystals were solubilized overnight in SDS buffer (SDS in 0.01 N HCl). Absorbance was recorded on a microplate reader (BioTek ELx800, Winooski, VT, USA) at 570 nm wavelength. The data were represented as a percentage in growth inhibition of cells treated with heated fiber versus cells grown in control medium (marked as 100%).

BrdU Incorporation. The influence of water extract of heated fiber on DNA synthesis within the cell was measured by colorimetric immunoassay, based on BrdU (analogue of thymidine, 5-bromo-2′-deoxyuridine) incorporation. C6 glioma cells were seeded onto 96-well microplates at a density of 2×10^3cells/well and incubated for 24 h in standard conditions. Next, the medium was discarded, and cells were exposed to fresh medium alone or with indicated concentrations of the extract (10, 50, 100, 250, 500, 1000 μg/mL). After 48 h of incubation BrdU was added, and following steps were performed according to manufacturer's procedures (cell proliferation ELISA BrdU; Roche Diagnostics GmbH, Penzberg, Germany). Absorbance was measured 450 nm wavelength using microplate reader (BioTek ELx800, Winooski, VT, USA).The effect of the extract on DNA synthesis was represented as a percentage in inhibition of BrdU incorporation versus control cells (marked as 100%).

Cytoskeleton Visualization. Alternations in the organization of microfilaments within the cells treated with water extract of heated fiber extract were shown by F-actin filament staining with use of rhodamine-conjugated phalloidin (RHPH). Cells grown on eight-well chamber slides (Nunc) were subjected to heated fiber extract in concentration 1000 μg/mL for 24 and 48 h. Then cells were rinsed twice with PBS and fixed with 3.7% paraformaldehyde for 20 min, followed by 5 min permeabilization in 0.1% Triton X-100. Next, half-hour incubation with phalloidin-rhodamine (1 μg/mL) was performed. Nuclei were counterstained for 5 min with Hoeschst 33342 (0.24 μg/mL). Cell images were captured with fluorescence microscopy (Olympus BX51 System Microscope; Olympus Optical Co., Ltd., Tokyo, Japan, and CellFamily AnalySIS software) at 400× magnification.

Immunobloting. Cells after treatment were washed with ice-cold PBS, harvested, and lysed for 1 h in ice-cold lysis buffer consisting of 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM EGTA, 1 mM Na_3VO_4, 20 mM NaF, 0.5 mM DTT, 1 mM PMSF, and protease inhibitor cocktail in PBS, pH 7.4. After centrifugation in 4 °C (14000g for 10 min) supernatants were collected and solubilized in 6× Laemmli sample buffer (0.5 M Tris-HCl, pH 6.8, 30% glycerol, 10% SDS, 5% β-mercaptoethanol, 0.012% bromophenol blue). Twenty micrograms of total protein was separated by SDS−PAGE (10−12% SDS−polyacrylamide gel) and then transferred into PVDF (polyvinyl difluoride) membrane (Millipore, Billerica, MA, USA). The blots were probed overnight with indicated primary antibodies, which were then detected with HRP-conjugated secondary antibodies. The visualization of the proteins was performed using an enhanced chemiluminescence detection system (Pierce, Rockford, IL, USA).

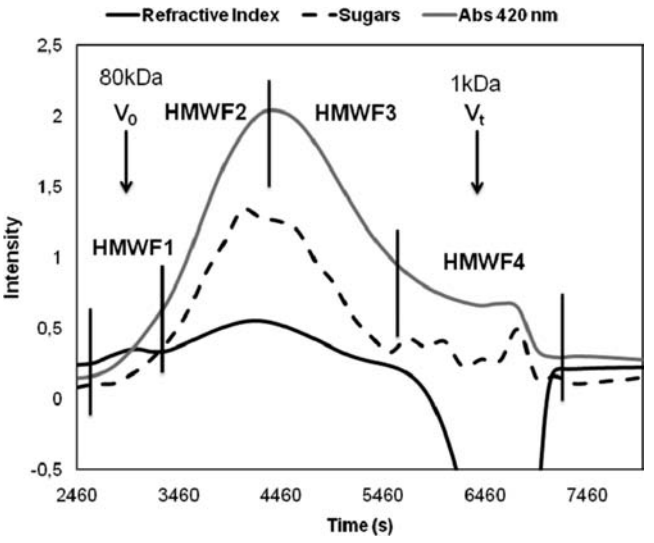

Figure 1. Chromatogram of the purification of HMW Potex by size-exclusion chromatography on Sephacryl S-200 HR. Fractions 2 and 3 were used for examination of antiproliferative activity.

Cell Cycle Analysis (DNA versus RNA Staining). The method of differential staining of cellular DNA and RNA is based on the use of metachromatic fluorochrome acridine orange (AO). Cells were detached from flask by trypsin solution, suspended in culture medium, and treated with a solution containing nonionic detergent Triton X-100 (Sigma) at low pH to permabilize the plasma membrane. Then the cells were stained with AO under specific ionic conditions, ensuring that cellular DNA is double stranded and stains orthochromatically emitting green fluorescence, while RNA subjected to denaturation becomes single stranded and fluorescens red ("red metachromasia"). Following AO staining the cellular red and green fluorescence was measured using FACSCalibur flow cytometer (Becton Dickinson, USA) with standard settings for green (FL1) and red (FL3) fluorescence detection. Details of the procedure are described elsewhere.[21,22]

Statistical Analysis. The data were plotted as the mean ± SD. ANOVA with Tukey post hoc test and column statistics were used for comparisons (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

RESULTS

Water Extract of Heated Potato Fiber Potex Contains Melanoidin Complexes. The roasting procedure rendered a brown Potex fiber, from which nearly 9% was rendered soluble by water extraction at room temperature. This material was also deep brown and was composed by nearly 43% of high molecular weight material (HMW Potex) isolated by ultrafiltration (MW cutoff 10 kDa). When purified by size exclusion chromatography (SEC) on Sephacryl S 200 HR, the majority of the material had a molecular weight lower than 80 kDa, and only a small amount of material eluted in the exclusion volume. The material eluted in the inclusion volume (Figure 1) and eluted as an almost homogeneous peak, and the total material (measured by the refractive index) was perfectly followed by the brown color (measured at 420 nm). Also, when the total sugars were measured in the collected fractions, its elution profile almost perfectly match the brown color. Four fractions were collected (Figure 1), but only two of them were tested for the antiproliferative activity.

Inhibition of C6 Glioma Cell Proliferation. Several concentrations of water extract of heated fiber AM4 (10−1000 μg/mL) were applied for 96 h of incubation to study growth inhibition in

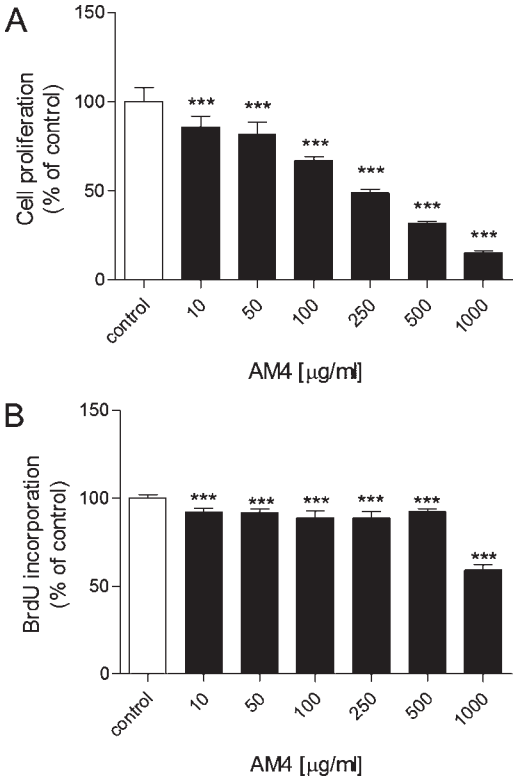

Figure 2. Inhibition of C6 glioma cell proliferation by water extract of heated potato fiber Potex (AM4). Cells were exposed to indicated concentrations of the extract for 96 h. Cell proliferation was measured by means of MTT test using tetrazolium salt as a substrate (A). C6 glioma cells were treated with various concentrations of the extract for 48 h. Quantification of cell proliferation was performed using colorimetric immunoassay, based on the measurement of BrdU incorporation during DNA synthesis (B). The values were means ± SD, $n = 6$. *** indicates $P < 0.001$ versus control cells (one-way ANOVA test, post hoc Tukey).

C6 rat glioma cells. Cell proliferation was determined by means of MTT method. All assayed concentrations of water extract of heated Potex decreased cell proliferation, and the effect was dose dependent (Figure 2A) with IC_{50} value of 212.4 μg/mL. At concentration 1000 μg/mL the extract reduced C6 cell proliferation down to 15.33% compared to nontreated cells.

To reveal the influence of Potex extract on DNA synthesis within the cells, C6 rat glioma cells were treated for 48 h with increasing concentrations of fiber extract (5−1000 μg/mL) followed by incubation with 5-bromo-2-deoxyuridine (BrdU). Incorporation of this synthetic analogue of thymidine was significantly affected in cells treated with the extract versus control cells (Figure 2B). Meaningful reduction of BrdU incorporation was observed only at concentration 1000 μg/mL and came to 59% compared with nontreated cells.

High Molecular Weight Components (HMW) Present in Water Extract of Heated Fiber Are Responsible for the Inhibition of C6 Cell Proliferation. High (HMW) and low (LMW) molecular weight component fractions obtained from water fiber extract by diafiltration were also tested for antiproliferative activity in C6 glioma cells with use of MTT assay. The comparison of their growth reducing effects with those exerted by initial extract showed stronger antiproliferative activity of high molecular weight components (HMW, IC_{50} = 115.8 μg/mL) than the extract (AM4). Moreover, statistically significant

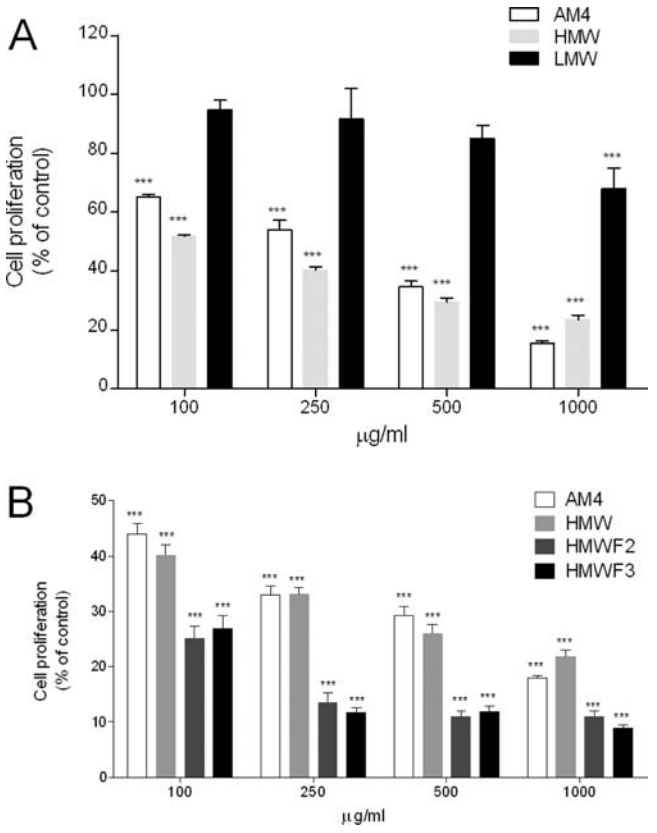

Figure 3. Compounds present in high molecular weight component fraction (HMW) rather than in low molecular weight component fraction (LMW) are responsible for the inhibition of C6 glioma cell proliferation caused by heated fiber water extract. Cells were exposed to following concentrations of initial extract (AM4), HMW and LMW fraction (A) or initial extract, HMW, HMWF2, and HMWF3 fractions (B) for 96 h. Cell proliferation was measured by means of MTT test using tetrazolium salt as a substrate. The values were means ± SD, $n = 6$. *** indicates $P < 0.001$ versus control cells (one-way ANOVA test, post hoc Tukey).

(vs control) reduction of cell proliferation by low molecular weight components (LMW) was not observed until the dose of 1000 μg/mL and was still much weaker compared to initial extract and HMW activities (Figure 3A). Further fractionation of HMW by size exclusion chromatography delivered four fractions, and two of them (HMWF1 and HMWF4) were excluded from the examination due to incomplete dissolving in culture medium. Remaining HMWF2 and HMWF3 fractions were tested for antiproliferative activity and revealed significantly higher potential to decrease proliferation of tested cells (IC_{50} value of 7.4 and 21.6 μg/mL, respectively) in comparison with initial extract and HMW fraction (Figure 3B).

Water Extract of Heated Potato Fiber Influenced the Morphology of C6 Glioma Cells. To further explore the growth modulatory effect of heated Potex and determine its impact on general cell condition, rhodamine-conjugated phalloidin staining was performed. F-actin filaments were visualized, and its organization was shown to be altered in cells treated with fiber extract. Moreover, intercellular connections typical for C6 glioma cell growth were found to be impaired after incubation with heated fiber (Figure 4).

Water Extract of Heated Potato Fiber Modulates Phosphorylation Status of MAPK and MAP Kinases in C6 Glioma Cells. We examined the influence of heated fiber extract (AM4) on the activation status of three major MAP kinases: ERK1/2 (extracellular signal-regulated kinase), p38, and SAPK/JNK (c-Jun N-terminal kinase) (Figure 5). In order to evaluate the effect of tested extract on the ERK pathway kinase activity, Western bloting was performed using phospho-specific antibodies detecting active form of the kinases. Significant inhibition of MEK1/2 phosphorylation (upstream activator of ERK1/2) was noted in cells treated with 1000 μg/mL heated fiber extract for all indicated periods of time. Even though, decreased ERK1/2 kinase phosphorylation was observed after 30 min and 1 h treatment, but the effect was diminished in cells incubated with the extract for 3 h. The inhibition was restored after 24 h (Figure 5A). Then, to examine if tested extract reduces activity of ERK kinases in dose-dependent manner, C6 cells were subjected to following concentrations of the extract (100, 250, 500, and 1000 μg/mL) for 1 h. On the lower panel of Figure 5A dose-dependent inhibition of ERK1/2 phosphorylation status is shown. The effect was observed already after treatment with 100 μg/mL fiber extract.

Next, the ability of heat-treated fiber extract to influence the activity of p38 and SAPK/JNK kinases was studied. Tested extract (1000 μg/mL) administration for indicated periods of time resulted in increased phosphorylation of both kinases (Figure 5B). The most intense effect was observed after only 30 min of incubation.

The Akt Kinase Activation Status in C6 Glioma Cells Is Impaired after Treatment with Heated Potato Fiber Extract. Protein kinase B (PKB, Akt) constitutes another potential target for malignant glioma treatment, due to its elevated expression in these cells. We explored changes in phosphorylation level of Akt kinase in C6 cells after incubation with fiber extract. Time- and dose-dependent effect has been determined with use of Western blot analysis. Figure 6 shows that fiber extract in concentration 1000 μg/mL markedly reduced Akt phosphorylation at Ser^{473}, which is indicative of lowered kinase activity. The effect was visible for a notable period of time and lasted from 30 min to 48 h of incubation with tested extract. Simultaneously, any changes in Thr^{308} phosphorylation were evoked when cells were subjected to heated fiber extract (data not shown).

Water Extract of Heated Potato Fiber Arrests C6 Glioma Cells in G_1 Phase of Cell Cycle. In order to explore other mechanisms by which fiber extract decreases cellular proliferation, we evaluated its influence on cell cycle progression. Rat glioma cells were incubated for 24 and 48 h in the presence of tested extract in concentrations 500 and 1000 μg/mL and then subjected to flow cytometry analysis. As shown in Figure 7A impact on cell cycle progression and accumulation of cells in G_1 was observed. The 24 h exposure to 1000 μg/mL extract resulted in increased number of cells in G_1 up to 75% compared with control conditions of 63% and decreased cell number in S phase up to 11.7% (control of 20.5%) or in G_2M up to 13.3% (control of 16,.5%). The prolonged (48 h) incubation evoked similar effect (data not shown).

The next step of our study was to examine if potato fiber extract-induced cell cycle inhibition was elicited by influencing several G_1 regulatory proteins. GSK-3 kinase (glycogen synthase kinase 3β) is a downstream target of Akt, which phosphorylated in specific residues becomes inactive. Active, unphosphorylated form of GSK-3 can act as a cell cycle regulator influencing stability of several molecules engaged in cell cycle progression, like cyclin D1 or inhibitor of cell cycle p21 protein. In our study, extract of heated fiber (dose 1000 μg/mL)

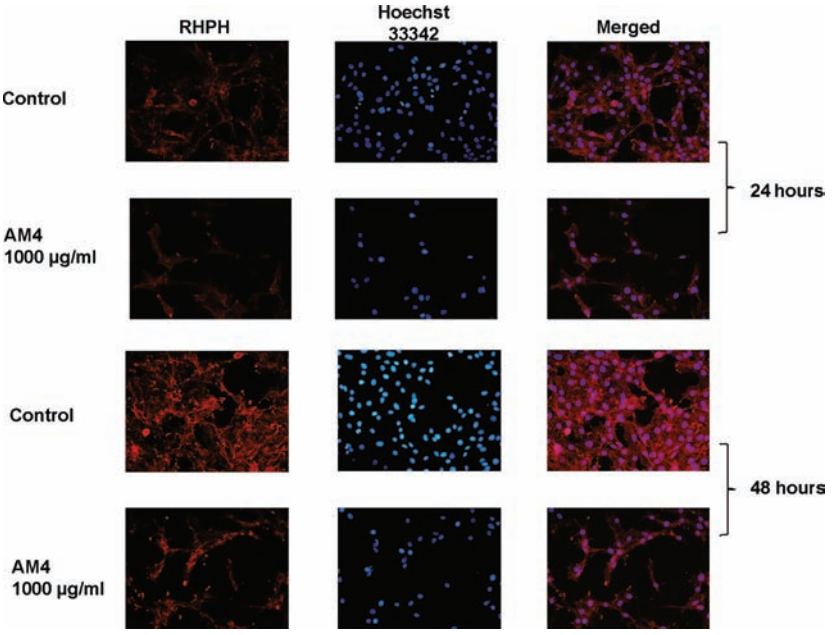

Figure 4. Heated Potex extract affects morphology of C6 glioma cells. Cells were grown in the presence of medium alone or tested extract for 24 or 48 h. Cells were then fixed and analyzed by rhodamine-conjugated phalloidin RHPH (red) staining. Nuclei were counterstained with Hoechst 33342 (blue). Fluorescence microscopy was performed to visualize cells at 400× magnification.

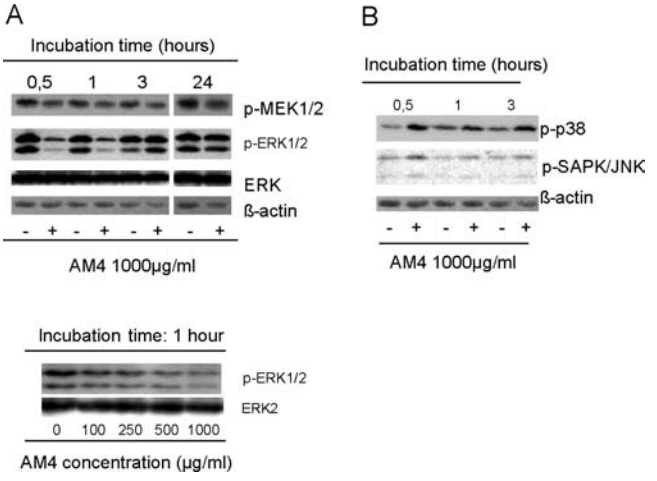

Figure 5. The influence of water extract of heated potato fiber Potex (AM4) on the activity of multiple MAPK pathway proteins. C6 glioma cells were treated with 1000 μg/mL extract for various periods of time (A, upper panel, and B) or with different concentrations of fiber extract (100, 250, 500, and 1000 μg/mL) for 1 h (A, lower panel). Harvested cells were lysed as described in Materials and Methods, and 20 μg of total protein was separated by SDS−PAGE followed by Western blot analysis using antibodies against phospho-MEK1/2 ($Ser^{217/221}$), phospho-ERK1/2 (Thr^{202}/Tyr^{204}), total ERK2, phospho-p38 (Thr^{180}/Tyr^{182}), and phospho-SAPK/JNK (Thr^{183}/Tyr^{185}). Blots were then reprobed with anti-β-actin antibody as internal control. Representative Western blot out of three independent experiments is shown.

reduced the phosphorylation of GSK-3β in Ser^{9} after 24 and 48 h of coincubation (Figure 7B). Since GSK-3 was shown to affect stability of cyclin D1, we explored levels of this protein after extract treatment, and decreased amounts of cyclin D1 were observed versus control. Simultaneously, levels of p21 protein were elevated in C6 cells due to 24 and 48 h exposition to tested extract (Figure 7B).

High Molecular Weight Components of Heated Potato Fiber Extract Are Responsible for Its Inhibitory Effect on ERK1/2, Akt, and GSK-3β Kinase Phosphorylation. In consistence with data obtained in MTT assay concerning the activity on cellular level, Western blot analyses enabled the observations that alternations at molecular level caused by water extract of heated fiber are mainly attributed to HMW compounds present in tested extract. Phosphorylation status of ERK1/2, Akt, and GSK-3β kinases was compared in C6 cells grown for 1 h with whole fiber extract, HMW fraction, and LMW fraction or in control conditions. All tested substances resulted in reduced phosphorylation of indicated kinases, although the effect after LMW treatment was much weaker than after initial extract or HMWC coincubation (Figure 8). Moreover, exposition to whole extract (AM4) as well as to high molecular weight components (HMW) resulted in similar pronounced effect, suggesting that majority of extract activity is caused by these HMW compounds.

DISCUSSION

Fiber, due to its prohealthy activities, is highly recommended nowadays for daily consumption. It is assumed to prevent colorectal cancer in several mechanisms, like decreasing transit time of stool bulk, binding secondary bile acids, and promoting the growth of beneficial microflora (i.e., bifidobacteriae) in the gut.[23,24] Moreover, dietary fiber is also considered as a protective food compound against cardiovascular diseases[25] and obesity.[26] Potex is a potato fiber preparation produced from the cell walls of potato tubers which is broadly used in food, mainly meat but also bakery industry. Potex comprises a number of strongly desirable properties, including high water and fat holding capacity, thus leading to its application as an ingredient to meat, milk, and bakery products improving their texture and taste. Since Potex-containing foodstuffs are often subjected to heat processing (like baking, cooking, or roasting), new compounds are formed. As Potex constitutes potato fiber rich in polysaccharides and proteins,

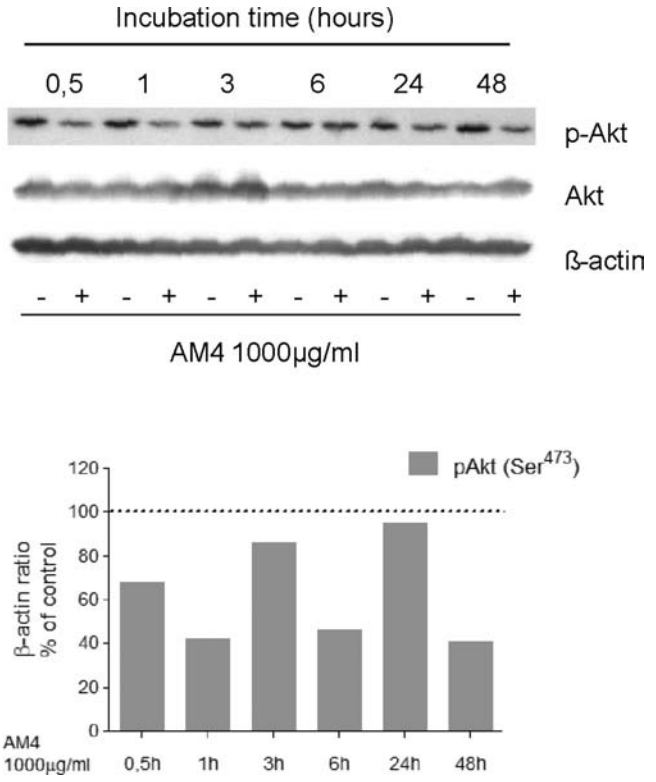

Figure 6. Inhibitory effect of heated fiber extract on protein kinase B (PKB, Akt) activity in C6 glioma cells. C6 glioma cells were treated with 1000 μg/mL extract for indicated lengths of time. Harvested cells were lysed as described in Materials and Methods, and 20 μg of total protein was separated by SDS−PAGE followed by Western blot analysis using antibodies against phospho-Akt (Ser^{473}) and total Akt. Blots were then reprobed with anti-β-actin antibody as internal control. Representative Western blot with densytometric analyses out of three independent experiments is shown.

melanoidins may form during its heat treatment. Free radical scavenging potential of melanoidins were broadly studied recently,[27−30] while few data indicating tumor cell growth-inhibiting effect are available. In the present study, we used the water extract of heated potato fiber Potex, as well as its fractions, to evaluate their influence on growth, proliferation, and cell cycle progression of glioma cells *in vitro*, using model rat C6 glioma cell line.

First step of our study was the evaluation of the inhibitory potential of heated potato fiber extract on glioma C6 cell growth. Concentration-dependent decrease in cell proliferation was observed. Markedly stronger cell growth inhibition after treatment with HMW fraction compared with LMW fraction suggests that extract's high molecular weight components are responsible for this cell proliferation repressing effect. Nevertheless, merely slightly reduction in DNA synthesis in the presence of lower than 1000 μg/mL doses of fiber extract was demonstrated. It indicates involvement of other than DNA synthesis regulation mechanisms, in the inhibitory activity of tested extract.

In many cancer cell systems, MAPK signaling is found to be affected, leading to increased cell growth, division, and tumor cell cycle progression.[31] First explored and currently the best studied mammalian MAPK pathway is extracellular signal-regulated protein kinase (ERK) pathway. It is involved in a multitudeof cellular processes, like proliferation, differentiation, migration, and apoptosis.[32] Growth factor-induced ERK signaling may also lead to disregulation of the cell cycle, since continuous activation of the kinase is necessary for S phase entry. Permanent ERK pathway induction is essential for cyclin D1 expression, as well as downregulation of proliferation inhibiting genes.[33] ERK 1/2 (p44/42) kinases become active when phosphorylated by dual-specifity protein kinases MEK 1/2 at residues Thr202/Tyr204 and Thr185/Tyr187, respectively.[34] Here, both MEK 1/2 and ERK 1/2 phosphorylation inhibition was observed in C6 glioma cells treated with heated potato fiber extract, thus suggesting its antiproliferative activity. Simultaneously, increased phosphorylation of p38 and SAPK/JNK proteins confirms cell proliferation inhibiting effect. The p38 MAPK, despite its role in response to environmental stresses and inflammation, acts as a suppressor of tumor development.[35] It inhibits cell proliferation, induces differentiation, may negatively regulate cell cycle, and is classically linked to apoptosis induction.[36,37] Thus, shown cell cycle cessation in glioma cells grown in the presence of heated potato fiber extract may also be partly attributed to p38 kinase activation. Upregulated p38 MAPK was reported to restrict cells in G1/S through p53 activation, which in turn induces p21 accumulation.[38] Direct stabilization of p21 inhibitory protein by p38 and JNK was also demonstrated.[39] Moreover, p38 may influence levels of cyclin D1 within the cells, indirectly repressing its gene expression[40] or directly phosphorylating the protein, thus leading to its ubiquitin-dependent degradation.[41] Likewise, SAPK/JNK may also play as a tumor suppressor.[42] Activated JNK MAPK was referred to increased stability and transcriptional activity of p53, a protein shown to act as a direct substrate for JNKs.[43]

Akt kinase, also known as a PKB (protein kinase B), is a serine/threonine protein kinase, which is crucial for cell proliferation, survival, glucose metabolism, protein synthesis, and response to growth factors. In cancer cells, constitutively activated Akt can lead to cell cycle progression, increased malignancy, and angiogenesis or inhibition of apoptosis. As a key mediator of signal transduction cascades it is often considered as a target for anticancer therapy.[44,45] Phosphorylation of Ser 473 and Thr 308 is required for full activation of PKB. Our data indicate that heated potato fiber Potex decreased the activity of Akt protein kinase, due to its impaired phosphorylation at serine 473. Since observed effect on Akt phosphorylation status was prominent and sustained, we conclude that majority of the antiproliferative activity exerted by tested extract can be ascribed to Akt kinase inhibition. Furthermore, the kinase was found to be upstream negative regulator of GSK-3 kinase. The protein, apart from its central role in glycogen as well as protein synthesis, may also induce cell cycle arrest in G1/S phase. When phosphorylated at Ser 9 due to Akt activity, GSK3β becomes inactive which prevents cyclin D1 degradation and causes cell cycle progression.[46] In a present study, heated fiber led to GSK-3β dephosphorylation following Akt kinase inactivation. Thus, observed decrease in cyclin D1 levels may also be attributed to elevated activity of GSK-3β protein. Although phosphorylated glycogen sythase kinase 3 was shown to lower the amount of p21 protein,[47] our study revealed raised levels of this cell cycle inhibitor. It indicates other molecular pathway leading to this change not involving action of GSK-3 kinase. Nevertheless, noted cyclin D1down-regulation with concomitant p21 upregulation points out cell cycle inhibitory activity of heated potato fiber. What more, it is in agreement with results obtained in flow cytometry cell cycle analysis, which showed impaired switch between G1 and S phases in glioma cells treated with the extract.

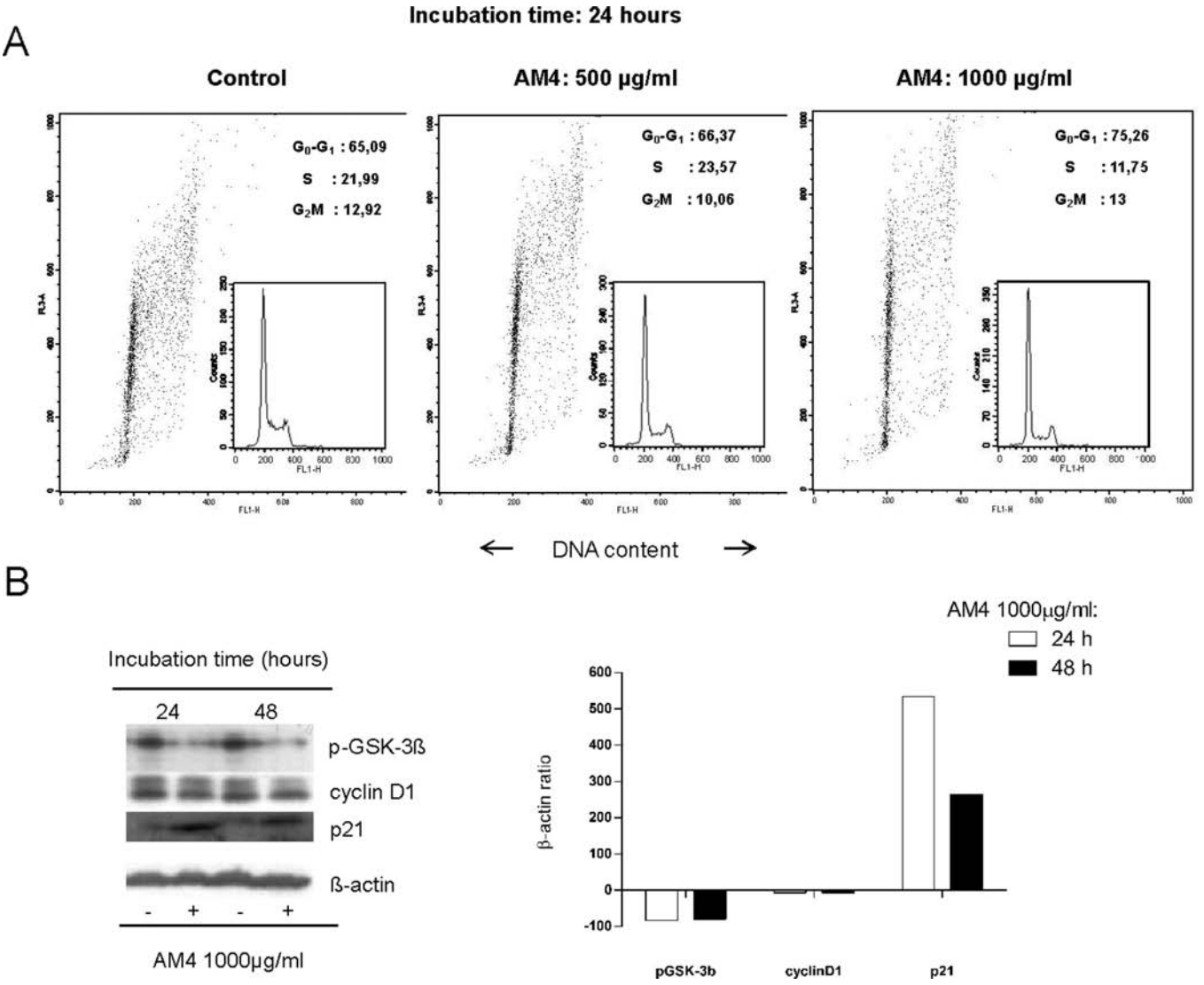

Figure 7. Extract of heated potato fiber arrests the cell cycle at G_1 in C6 glioma cells. Cells were treated with 500 or 1000 μg/mL fiber extract. Flow cytometry analyses were performed after 24 h of incubation, and DNA and RNA content was analyzed. The percentages of cell population in each phase of the cell cycle are included in each plot. Representative histograms and dot plots from three independent experiments are shown (A). C6 cells were grown in the presence or absence of the extract (1000 μg/mL) for 24 and 48 h (B). Harvested cells were lysed as described in Materials and Methods, and 20 μg of total protein was separated by SDS−PAGE followed by Western blot analysis using antibodies against phospho-GSK-3β (Ser^9), cyclin D1, and p21. Blots were then reprobed with anti-β-actin antibody as internal control. Representative Western blot with densytometric analyses out of three independent experiments is shown.

Fractionation of initial extract of heated potato fiber Potex resulted in high (HMW) and low (LMW) molecular weight fraction isolation. In consistence with our expectations, the extract was revealed to contain high molecular weight brown compounds, i.e., melanoidins. The elution pattern obtained by SEC, regarding the brown color followed closely by the sugars, resembles the elution behavior of the melanoidins from coffee infusions.[48] In fact, the HMW Potex due to its high molecular weight and brown color can be regarded as a melanoidin complex.[49] Furthermore, present study indicates these components as major causatives of glioma cell growth-inhibiting activity exerted by tested extract. To address the mechanism of antiproliferative activity elicited by heated Potex to its specific components, we investigated initial extract, as well as HMW and LMW fractions toward glioma cell growth inhibition. Experiments on both cellular and molecular level provided data confirming significantly stronger activity of HMW versus LMW compounds. Our results seem to be sparse, regarding cancer cell inhibitory properties of melanoidins. However, Kamei et al. reported tumor cell growth repressing activity of melanoidins obtained from miso and shoyu.[50] Marko et al. showed antitumor potential of a number of melanoidins formed under household heating conditions. Like our findings, these Maillard browning products were revealed to switch of MAPK signaling pathway, cause cell cycle arrest, and affect microtubule organization within the cells.[51,52]

In conclusion, the data reported here indicate the inhibitory effect of water extract obtained from heated potato fiber Potex on growth of C6 glioma cells *in vitro*. It was revealed that observed activity of tested extract was linked with its ability to decrease cell proliferation and to impair cell cycle progression. Cell morphology was also affected after exposure to heated fiber extract. In consistence with results emerging from the comparison of several

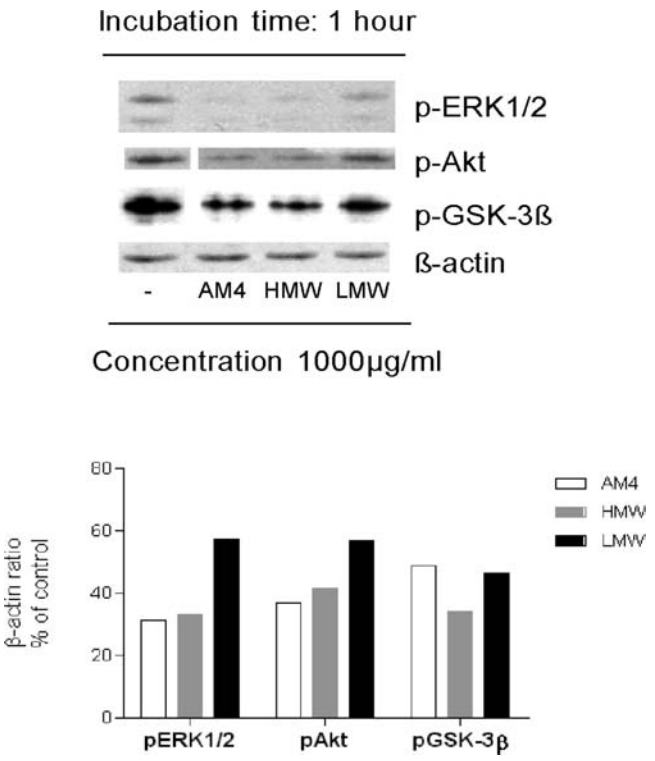

Figure 8. High molecular weight components present in heated potato fiber extract are responsible for decreased phosphorylation of several kinases. C6 glioma cells were treated with 1000 μg/mL initial heated potato fiber, HMW and LMW fractions for 1 h. Harvested cells were lysed as described in Materials and Methods, and 20 μg of total protein was separated by SDS−PAGE followed by Western blot analysis using antibodies against phospho-ERK1/2 (Thr^{202}/Tyr^{204}), phospho-Akt (Ser^{473}), and phospho-GSK-3β (Ser^{9}). Blots were then reprobed with anti-β-actin antibody as internal control. Representative Western blot with densytometric analyses out of three independent experiments is shown.

heated Potex fractions, we postulate that exerted potential is mainly caused by melanoidin complexes present in the extract. Further study, including broad range of cancer models, is needed to verify anticancer potential of heated Potex and possibility of its future application as a functional food ingredient and chemopreventive agent.

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ABBREVIATIONS USED

AM4, water extract of heated (180 °C/120 min) Potex; BrdU, 5-bromo-2-deoxyuridine; ERK, extracellular signal-regulated kinase; GSK-3β, glycogen synthase kinase 3β; HMW, high molecular weight fraction; IC_{50}, half-maximal inhibitory concentration; JNK, c-Jun N-terminal kinase; LMW, low molecular weight fraction; MAPK, mitogen-activated protein kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; RHPH, rhodamine-conjugated phalloidin; SAPK, stress-activated protein kinase.

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