Natural microbial polysaccharide sulphoethyl glucan as antigenotoxic and cancer preventing agent*

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Naturally occurring polysaccharides isolated from the yeasts are the substances with versatile intriguing biomodulatory activities. One of the novel derivatives prepared from the (1→3)-β-D-glucan isolated from the cell walls of baker's yeast *Saccharomyces cerevisiae* is sulfoethyl glucan (SEG). Its DNA-protective, antimutagenic, anticlastogenic and cytotoxic/cytostatic enhancing effect was evaluated using five eukaryotic systems. SEG showed bioprotective effect in recombination-repair-deficient strain of alga *Chlamydomonas reinhardtii* against methyl methanesulfonate-induced genotoxicity, antimutagenic effect against ofloxacin-induced genetic changes in yeast *Saccharomyces cerevisiae* assay and anticlastogenic activity in plants *Vicia sativa* and *Vicia faba* assays against maleic hydrazide-induced clastogenicity. In the combined application with cytostatic drug vumon, SEG exerted enhancement of the drug's cytotoxic/cytostatic effect in the cell revitalization assay using mouse leukemia cells. The study sheds light on the possible mechanisms of actions and utilization of this microbial polysaccharide derivative in the cancer prevention and therapy.

Key words: yeast polysaccharide, sulphoethyl glucan, antigenotoxicity, cancer prevention and therapy, microbial and plant assays, cell revitalization assay

In the recent decades, much data has been collected on various naturally occurring compounds and their ability to protect against certain types of mutagens and carcinogens. These substances are often included into the group of chemopreventive substances [1]. A rational use of chemopreventive agents is based not only on the assessment of their efficacy and safety but also on understanding of their mechanisms of action. A detailed classification is proposed which covers variety of mechanisms interfering with different phases of mutagenesis and carcinogenesis. Several mechanisms such as inhibition of genotoxic effects, antioxidant activity and scavenging of free radicals, inhibition of cell proliferation and signal transduction modulation may be involved [2–5]. Such compounds also include microbial polysaccharides which are located both inside the fungal cell walls or on the fungal cell surfaces, and possess marked immunological properties ranging from non-specific stimulation of host immune system, resulting in antitumor, antiviral, and anti-infective effects, to antioxidant, antimutagenic, or hematopoetic activity [6–8].

On the basis of their localization and function in the microbial cell wall, polysaccharides can be divided in the skeletal polysaccharides, polysaccharides of amorphous matrix, and the cell-surface polysaccharides. In the fungal cell walls, β-D-glucans are the major skeletal constituents that ensure the rigidity and define morphological properties of the cell. Matrix and cell-surface polysaccharides are amorphous homo- and heteropolysaccharides (e.g., mannans or glucomannans), and are often associated with proteins. Due to their ability to modulate host immune system, β-D-glucans isolated from fungi, lichens, algae, and bacteria are assigned to the class of drugs known as biological response-modifiers (BRMs) [9]. Some other fungal polysaccharides, e.g. glucomannan from *Candida utilis* reveal similar immuno-

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modulating properties [7]. Recently obtained data strongly support the assumption that microbial polysaccharides mediate their protective and immunopotentiating effect by binding to specific sites (receptors) on monocytes/macrophages and granulocytes triggering a cascade of immunological events. Microbial polysaccharides play role of signaling molecules for innate immune system through the pathways involving stimulation of several cytokines and kinases, which in the end results in general augmentation of immune defense [10].

The main immunopharmacological activities of β-D-glucans and glucomannans include: increase the host resistance to viral, bacterial, fungal, and parasitic infections, radioprotection and hematopoetic stimulation. Some fungal glucans and mannans revealed antioxidant, antimutagenic and antitumor activities [11–17].

In the present paper, we describe the results of assessment the ability of a water soluble derivative sulfoethyl glucan (SEG) prepared from the yeast cell wall to protect DNA and to enhance therapeutic effect of cytostatic drugs.

Material and methods

Chemicals. Methyl methanesulfonate (MMS) and ofloxacin (OFX) were purchased from Sigma (St. Louis, MO). Maleic hydrazide (MH) was obtained from Merck (Darmstadt, Germany) and vumon (VM 26) was obtained from Bristol-Myers Squibb GmbH (Munich, Germany).

Preparation and characterization of SEG. The water-insoluble (1→3)-β-D-glucan was isolated from the commercial baker's yeast biomass purchased from Slovlik (Trenčín, Slovak Republic). Yeast cells were treated with 6% NaOH at 60 °C followed by 4% phosphoric acid extraction at room temperature as previously described [18]. After the removal of all soluble material, β-D-glucan was left as the insoluble residue. Sulfoethylation of the glucan was performed according to the method described by CHORVATOVIČOVÁ et al [19]. The degree of substitution (DS) determined by potentiometric titration was 0.3 and the molecular mass determined by HPLC was 250 kDa.

Chlamydomonas reinhardtti bioprotectivity assay. The SEG bioprotectivity evaluation in the unicellular green alga *C. reinhardtii* was carried out in the wild type (WT) and recombination-repair-deficient strain *uvs10* (isolated at the Department of Genetics, Faculty of Natural Sciences, Comenius University, Bratislava). Algal cells were cultivated with a 12-h photoperiod in a liquid medium supplemented with acetate for 3–4 d (log growth phase) at 25 °C and harvested by centrifugation for 7 min at 3,000 rpm. Subsequently, algal cell suspensions were treated with SEG (0.1 mM), MMS (0.1, 0.2, and 0.3%), and simultaneously treated with SEG and MMS for 30 min in the dark. After washing cells were plated on Petri dishes with acetate medium. Survival was evaluated by microscopic method, assessing the number of living cells (colonies) and dead cells due to the cytotoxic and mutagenic (lethal mutations) effects of MMS as described by MIADOKOVÁ et al [20].

Saccharomyces cerevisiae mutagenicity/antimutagenicity assay. As a testing procedure the assay according to ZIMMERMANN et al [21] was used. *S. cerevisiae* D7 strain (*MATa/MATα, ade2-40/ade2-119, trp5-12/trp5-27, ilv1-92/ilv1-92*) enables simultaneously to detect toxicity, revetants at the *ilv1* locus, mitotic crossing-over and total aberrants at the *ade2* locus, as well as mitotic gene convertants at the *trp5* locus. Prior to each experiment, single colonies of the yeast D7 strain were tested for the frequency of spontaneous convertants at the tryptophan (*trp*) locus and revertants at the isoleucine (*ilv*) locus to select a culture with a low spontaneous frequency of these genetic changes. Exponentially growing cells ($2x10^7$ cells/ml) washed and resuspended in potassium phosphate buffer (0.1 M, pH 7) were treated with SEG (1 μM and 10 μM), OFX (250 μg/ml) and co-treated with SEG and OFX for 22 h at 28 °C with permanent shaking. After the treatment, washed cell suspensions were plated on appropriate media: $1–2x10^6$ cells/plate to detect revertants on a synthetic selective medium without ilv, $1–2x10^5$ cells/plate to detect convertants on a selective medium without trp, and $2x10^2–2x10^3$ cells/plate to detect survival, mitotic crossing-over, and total aberrants at *ade2* locus on synthetic medium. The plates were incubated at 28 °C for 5–12 days. Survival and all genetic changes were evaluated using a Biotran III Colony Counter (New Brunswick Scientific Co., Edison, NJ).

Vicia faba clastogenity/anticlastogenity assay. The cytogenetic test using *V. faba* L. was performed according to KANAYA et al [22] as modified by MIADOKOVÁ et al [23]. After 6 h of soaking in tap water at 22 °C, the seeds of *V. faba* were allowed to germinate for 4 d in moist sawdust. The seed coats of seedlings with primary roots 3–5 cm long were removed and shoots were shortened to stimulate the growth of lateral roots. The modified seedlings were incubated in aerated water for 4 d at 22 °C, and than treated for 2 h with SEG (0.1 μM), MH (25; 50, and 100 μM) applied separately or together. After the treatment, the seedlings were placed in tap water at 22 °C for a recovery period of 26 h in the dark, and the roots were fixed and permanent slides were prepared using a Feulgen method.

Vicia sativa phytotoxicity and clastogenity/anticlastogenity assay. The simultaneous phytotoxicity and clastogenity/anticlastogenity assay was performed with plant species *V. sativa* L. according to MURÍN [24]. After 24 h of soaking at 25 °C in the solutions of SEG (0.1 μM) and MH (25; 50, and 100 μM) used as a positive control, the seeds of *V. sativa* were allowed to germinate on Petri dishes (diameter = 18.5 cm) with filter paper soaked with the same solutions of SEG and MH as those used for seed soaking. Phytotoxicity was assayed after 72 h cultivation in the dark in a thermostat at 25 °C. The seedlings, in which the root growth was inhibited at least by 25, 50, and 75% were fixed and used for chromosome aberrations evaluation. The roots were fixed and per-

manent slides were prepared by the Feulgen method. In both assays chromosome aberrations were determined at least in 1000 ana-telophases.

Cell-revitalization assay. This assay enables to study the biomodulating effect of SEG on mouse leukemia cells (line P388D1) damaged by pre-treatment with a cytostatic drug VM 26. Mouse leukemia cell line P388D1 was obtained from American Type Culture Collection (Rockville, MD). Cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (Grand Island Biological Co., Grand Island, NY), 100 U/ml penicillin G, 100 µg/ml streptomycin, and 2 mM L-glutamine (Sebac, Aidenbach, Germany) in an atmosphere of 5% CO_2 in humidified air at 37 °C. The assay comprises two phases. In the first stage of the experiment, cells were exposed for 24 h to the treatment with commercial cytostatic VM 26 (0.06 µg/ml). This step was carried out in such a way that the drug treatment resulted in a complete proliferation arrest of the mouse leukemia cells. After 24 h, the cytostatic was washed out and leukemia cells were resuspended in a fresh medium with/without SEG (0.2 mg/ml). In the second stage of the experiment (post-exposure phase), viability of cells was evaluated by the Trypan blue exclusive assay during the following 7 days. The goal of the second step was to evaluate the progressive cell death or the recovery of cell growth (revitalization process of damaged cells) in a growing medium with or without SEG. The "negative" results in the cell-revitalization assay could be a good marker for cytotoxic/therapeutic treatment. Cell viability is a relative value calculated as a ratio of viable cells number assessed during 1–7 days cultivation to the viable cell number assessed on the first day of the second phase [25].

Statistical evaluation. In all assays used differences between the groups were analyzed using the standard *t*-test for paired observations. Statistical analyses were performed using ISI<R> Software, Version 2.0.

Results

Chlamydomonas reinhardtti bioprotectivity assay. Experimental results obtained after simultaneous treatment of algal recombination repair deficient strain *uvs10* with SEG and MMS are illustrated in Figure 1 (values represents the means from three experiments). Survival after MMS treatment documented a well known sensitivity of recombination-deficient-repair strains to alkylating agents. The results obtained showed that SEG increased the survival of MMS-treated algal cells at all three concentrations of MMS, however, a statistically significant bioprotective effect was proved only at the lowest concentration. SEG did not exerted bioprotective effect against MMS-induced genotoxicity in wild type cells (data not shown).

Saccharomyces cerevisiae mutagenicity/antimutagenicity assay. Data presented in Figures 2 and 3 (values represent the means from four experiments) demonstrate that antimutagenic effect of SEG against OFX-induced mutagenicity was clearly exhibited in *S. cerevisiae* assay. SEG applied alone did not increase the frequency of revertants at the *ilv1* locus and convertants at the *trp5* locus comparing to the negative control, while it significantly reduced the frequency of OFX-induced revertants and convertants at both concentrations used.

Vicia faba and Vicia sativa clastogenity/anticlastogenity assay. As demonstrated in Table 1 and 2, SEG itself did not reveal any clastogenic effect, while exerting apparent anticlastogenic effect against MH-induced clastogenicity at both assays used. SEG statistically significantly reduced the frequency of chromosomal aberrations at all MH concentrations applied.

Cell-revitalization assay. During the first phase of this assay, a complete cell proliferation arrest of the mouse leukemia cells was attained after VM 26 treatment. In an independ-

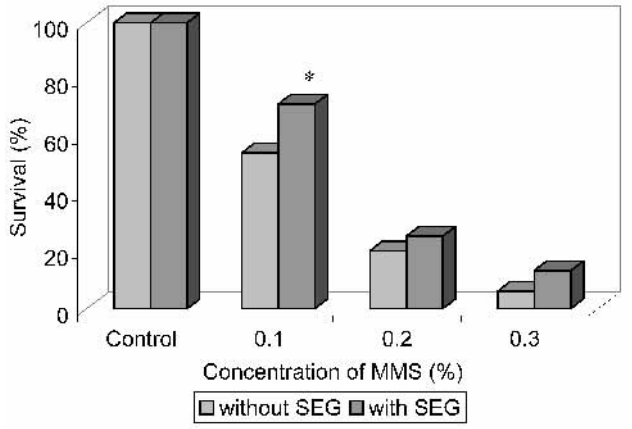

Figure 1. Bioprotective effect of SEG (0.1 mM) against MMS-induced genotoxicity in the *C. reinhardtii* recombination-repair-deficient strain *uvs10*. Control – H_2O. *Significant difference in comparison with MMS at p<0.05.

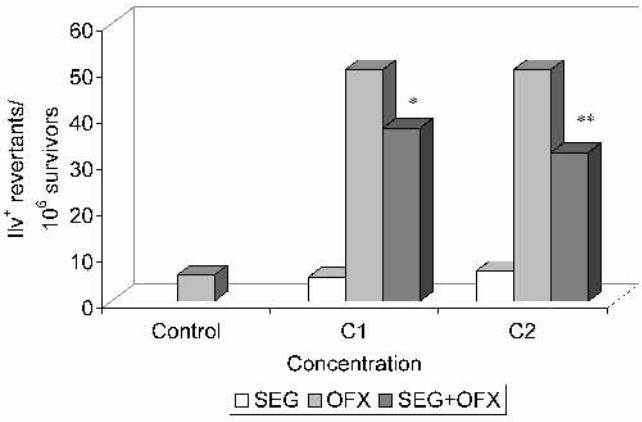

Figure 2. Antimutagenic effect of SEG against OFX-induced revertants in the *S. cerevisiae* D7 strain. Control – H_2O. C1 (SEG 1 µM, OFX 250 µg/ml). C2 (SEG 10 µM, OFX 250 µg/ml). *,Significant difference in comparison with OFX at p<0.05, p<0.01.**

ent experiment it was demonstrated that at a similar application SEG did not reveal any cytotoxic/cytostatic effect (the results not shown). During the second phase, the drug-pretreated cells (Fig. 4; values represent the means from five experiments) were incubated with or without SEG, and during 1–7 d of the post-exposure incubation the viability of cells was monitored. The significantly increased cytotoxic/cytostatic effect was observed when leukemia cells pretreated with VM 26 were cultivated in the presence of SEG. Cessation of the cell proliferation/cell revitalization of the drug-treated leukemia cells confirmed that the joint administration VM 26 and SEG was successful and therefore might be worth consideration for a more efficient anticancer therapy.

Table 1. Anticlastogenic effect of SEG against MH applied to *Vicia faba* L

Compound tested	Concentration µM	Total number of cells analyzed in ana-telophase	Number of cells with aberrations	Rate of aberrations (%)
Control		1204	4	0.33±0.17
SEG	0.1	1623	8	0.49±0.17
MH	25	1489	34	2.28±0.39^{++}
MH	50	1396	52	3.72±0.51^{++}
MH	100	1202	108	8.99±0.83^{++}
MH + SEG	25 + 0.1	1560	10	0.64±0.20**
MH + SEG	50 + 0.1	1299	20	1.54±0.34**
MH + SEG	100 + 0.1	1170	43	3.68±0.55**

Values are mean ±SD; control – Sorensen buffer, pH=6.2; $^{++}$Significant difference in comparison with control at $p<0.01$; **Significant difference in comparison with MH at $p<0.01$.

Table 2. Anticlastogenic effect of SEG against MH applied to *Vicia sativa* L

Compound tested	Concentration µM	Total number of cells analyzed in ana-telophase	Number of cells with aberrations	Rate of aberrations (%)
Control		1129	2	0.18±0.13
SEG	0.1	1681	5	0.29±0.13
MH	25	1289	59	4.58±0.58^{++}
MH	50	1021	179	17.53±1.19^{++}
MH	100	1047	235	24.16±1.32^{++}
MH + SEG	25 + 0.1	1432	42	2.93±0.45*
MH + SEG	50 + 0.1	1161	125	10.77±0.91**
MH + SEG	100 + 0.1	1099	176	16.01±1.11**

Values are mean ±SD; control – H_2O; $^{++}$Significant difference in comparison with control at $p<0.01$; *,**Significant difference in comparison with MH at $p<0.05$, $p<0.01$.

Discussion

The issue of changing the traditional cytotoxic/cytostatic therapy by combining convential chemotherapeutics with the natural compounds that may reveal enhancing effect at their simultaneous administration is very promising and deserves to be seriously considered. Certain synergy may occur between both types of bioactive compounds thus leading to a more efficient therapeutic outcome.

In the recent years, extensive investigations of the antioxidant and free radical scavenging activity of fungal cell wall polysaccharides have been carried out in a search of natural antioxidants which could be potentially used in the treatment of many diseases associated with the oxidative stress [12, 15, 17, 26–28]. Since it is now generally recognized that free radical damaging is implicated in many rheumatic, neurodegenerative, and oncological diseases [29, 30] the search for efficient antioxidants has significantly intensified. Fungal (1→3)-β-D-glucans demonstrated along with the immunomodulatory also a pronounced antioxidant activity (reviewed in

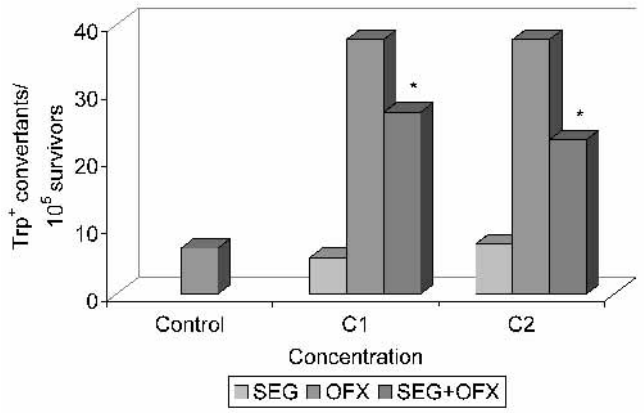

Figure 3. Antigenotoxic effect of SEG against OFX-induced convertants in the *S. cerevisiae* D7 strain. Control – H_2O. C1 (SEG 1 µM, OFX 250 µg/ml). C2 (SEG 10 µM, OFX 250 µg/ml). *Significant difference in comparison with OFX at $p<0.05$.

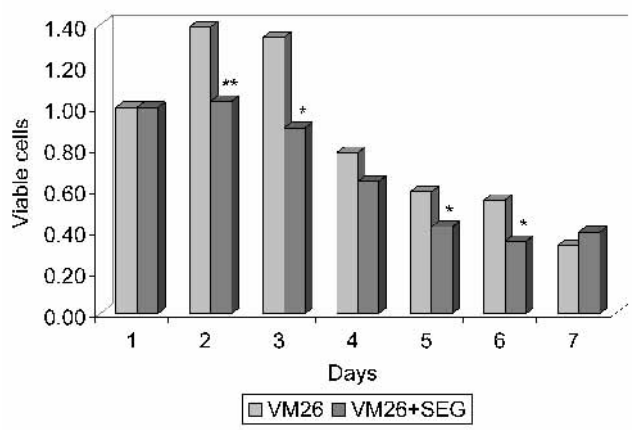

Figure 4. Cell revitalization assay on mouse leukemia cells P388D1. Suppression of viability after SEG administration to the VM 26 treated cells. *,**Significant difference in comparison with VM 26 at $p<0.05$, $p<0.01$.

[31]), however due to their general insolubility in water, chemical derivatization shoud be performed in order to prepare water-soluble derivatives suitable for biological *in vitro* and *in vivo* tests. SEG is one of such recently prepared derivatives of the β-D-glucan isolated from the baker's yeast *Saccharomyces cerevisiae*. In this study, diverse biomodulatory effects of SEG combined with several agents differing in the modes of action were established using five different model systems and relevant experimental techniques. The results obtained indicate existence of several possible biomodulatory mechanisms of SEG, which are extremely diverse (DNA-protective, antimutagenic, anticlastogenic, cytotoxic/cytostatic effect enhancing).

SEG exerted bioprotective effect against MMS-induced genotoxicity in recombination- repair-deficient strain *uvs10* of *Chlamydomonas reihardtii* (Fig. 1). This effect may indicate its role: in scavenging of reactive oxygen radicals, in inactivation of MMS by SEG acting as desmutagen, or in stimulation of non-damaged repair mechanism by SEG acting as biomutagen. In the previous work, we have established the bioprotective effect of SEG against MMS-induced genotoxicity in the excision-repair-deficient mutant *uvs12 C. reinhardtii* [14]. Thus, explanation of antigenotoxicity due to SEG acting in a desmutagenic manner along with its radical scavenging action is more probable.

Antimutagenic activity of SEG against OFX-induced genetic changes was proved in our experiments involving the yeast *Saccharomyces cerevisiae.* A decreased number of revertants and convertants (Figs. 2 and 3) may be the result of ability of SEG to prevent oxidative damage of DNA by reactive oxygen species (ROS) produced by OFX, which is known to be a DNA gyrase-inhibitor and producer of ROS [32]. Protective effects of SEG against oxidative DNA damage induced by hydrogen peroxide (H_2O_2) and visible light-excited methylene blue (MB) in V79 hamster lung cells has been recently described by SLAMEŇOVÁ et al [16]. Antioxidative and antimutagenic activity of SEG was also ascertained against OFX and acridine orange (AO) in the chloroplast DNA of the flagellate *Euglena gracilis* [27] and against 2-aminofluorene (2-AF) in the *Salmonella*/microsome assay (using *S. typhimurium* TA100 strain) [14].

From the currently available higher plant bioassays, the *Vicia faba* root tip meristem chromosomal aberration assay was chosen, as root tip cells of *Vicia faba* have been the most frequently used higher plant material for assessing chromosome damage as well as chromosome protection [22]. The simultaneous phytotoxicity and clastogenity/anticlastogenity assay was performed on another plant species *Vicia sativa* according to MURÍN [24]. It is generally known that MH acts as an antimetabolite in connection with synthesis and metabolism of nucleic acids. On the basis of the results obtained in the *V. faba* and *V. sativa* assays, significant anticlastogenic effect of SEG against MH-induced clastogenicity was proved (Tab. 1 and 2). Anticlastogenic action of SEG demonstrated in these plant assays is in accordance with the previously described reduction of cyclophosphamide-induced micronuclei in polychromatic erythrocytes of an animal model system (mouse bone marrow) mediated by SEG [19].

From the point of view of potential application of SEG in the cancer chemoprevention and combined chemotherapy-biotherapy experiments involving cell line cultures may render very important information. The biomodulatory effect of SEG was investigated in mouse leukemia cell line P388D1. We found that SEG applied to logarithmically growing cells increased the cell proliferation (data not shown). However, as can be seen from Figure 4, the post-exposure SEG treatment led to a decreased viability of cells pre-treated with a cytostatic drug VM 26. This fact could be explained by SEG's potential proliferation stimulating ability, which in case of the damaged cells could, abolish the arrest of cell cycle and accelerate the transition of not completely repaired cells to apoptosis [15, 33].

Our findings allow the conclusion that sulfoethylated water-soluble derivative of β-D-glucan isolated from yeast *Saccharomyces cerevisiae* is a substance having a broad range of biomodulatory properties (DNA-protective, antimutagenic, anticlastogenic, cytotoxic/cytostatic effect enhancing); however, its biological activity is dependent on the combined application with other biologically active compounds.

The observed activity of SEG in the cell revitalization assay, together with the results of the other assays indicate that this compound might be used, after performing the *in vivo* experiments, in the putative biomedical applications, since positive results have already been obtained when combining chemotherapeutic agents and other natural compounds.

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