Serbian Biochemical Society
Sixth Conference

"Biochemistry and Interdisciplinarity: Transcending the Limits of Field"

Proceedings

Faculty of Chemistry
Belgrade 2016

Serbian Biochemical Society

Proceedings

Editor: Ivan Spasojević
Technical secretary: Jelena Nestorov
Cover design: Zoran Beloševac
Publisher: Faculty of Chemistry, Serbian Biochemical Society
Printed by: Colorgrafx, Belgrade

CIP

Serbian Biochemical Society
Sixth Conference

with international participation

Faculty of Chemistry, University of Belgrade,
18.11.2016. Belgrade, Serbia

"***Biochemistry and Interdisciplinarity: Transcending the Limits of Field***"

PROGRAMME

10:00-10:10	Welcome message Mihajlo B. Spasić (President of the Serbian Biochemical Society)
10:10-10:40	Djuro Josić Division of Medicinal Chemistry, Department of Biotechnology, University of Rijeka, Rijeka, Croatia **Proteomics and glycomics as a tool to follow cell injury and malignant modification** (FEBS3+ Lecture)
10:40-11:00	Sanja Mijatović Department of Immunology, Institute for Biological Research "Siniša Stanković", University of Belgrade, Belgrade, Serbia **Differentiation *vs.* apoptosis - new concept in the treatment of aggressive cancers**
11:00-11:30	Coffee break
11:30-11:50	Milena Čavić Department of Experimental Oncology, Institute for Oncology and Radiology of Serbia, University of Belgrade **Dopamine and adenosine G protein-coupled receptor heteromers as pharmacological targets in neuroendocrine lung carcinoids**
11:50-12:10	Milan Zarić Institute of Biochemistry, Faculty of Medical Sciences, University of Kragujevac, Kragujevac, Serbia **The effects of bioactive compounds on viability of chronic lymphocytic leukemia lymphocytes**

12:10-12:30	Tamara B. Popović Centre of Excellence in Nutrition and Metabolism, Institute for Medical Research, University of Belgrade **Fatty acids in phospholipids as biomarkers of aging and pathological conditions**
12:30-12:50	Milena Despotović Department of Biochemistry, Faculty of Medicine, University of Niš, Niš, Serbia **Vitamin D receptor gene polymorphisms in inflammatory diseases**
12.50-13.20	Poster session
13.20-13.40	Cocktail
13:40-14:00	Suzana Jovanović-Šanta Department of Chemistry, Biochemistry and Environmental Protection, Faculty of Sciences, University of Novi Sad, Novi Sad, Serbia **Biomedical potential of modified steroids**
14:00-14:20	Jelena Nestorov Department of Biochemistry, Institute for Biological Research "Siniša Stanković", University of Belgrade **Fructose in the court of justice: good or bad sugar?**
14:20-14:40	Milan Žižić Department of Life Sciences, Institute for Multidisciplinary Research, University of Belgrade **Vanadium speciation detection by synchrotron based X-ray absorption spectroscopy: application in biological systems**
14.40-15:00	Discussion and concluding remarks

Poster Session

Ana Marija Balaž

Center of Chemistry, Institute of Chemistry, Technology and Metallurgy, University of Belgrade

Semi rational design of cellobiose dehydrogenase from *Phanerochaete chrysosporium* for increased oxidative stability

Sanja Berić

Faculty of Sciences, University of Novi Sad

Fractionation and characterization of lignans from *Anthriscus sylvestris* root extracts

Marija Blažić

Center for Chemistry, Institute of Chemistry, Technology and Metallurgy, University of Belgrade

Directed evolution of cellobiose dehydrogenase for higher activity

Barbara S. Janović

Department of Chemistry, Institute of Chemistry Technology and Metallurgy, University of Belgrade

Investigation of vitamin C effects on DNA damage during enzymatic decolorization

Jovana Jovankić

Department for Biology and Ecology, Faculty of Science, University of Kragujevac

Molecular mechanisms of redox status and antitumor activity of extracts of invasive plant species (*Robinia pseudoacacia* and *Amorpha fructicosa*) in MRC-5 and MDA-MB-231 cell lines

Milica Kojadinović

Centre of Research Excellence in Nutrition and Metabolism, Institute for Medical Research, University of Belgrade

Effect of ellagitannins on oxidative stress of colorectal adenocarcinoma cells Caco-2

Tatjana Majkić

Department of Chemistry, Biochemistry and Environmental Protection, Faculty of Sciences, University of Novi Sad

Chemical characterisation and biological potency of merlot variety grape juice and wine from Fruška Gora region

Miloš M. Matić

Department of Biology and Ecology, Faculty of Science, University of Kragujevac

Neuropeptide Y induces nitrosative stress and inhibits migration in human choriocarcinoma cell line JEG 3

Ana Medić

School of Medicine, University of Belgrade

Biodegradation of n-alkanes and polycyclic aromatic hydrocarbons by *Pseudomonas aeruginosa* san ai

Ivana Lj. Milenković

Department of Life Sciences, Institute for Multidisciplinary Research, University of Belgrade

***In vivo* toxicity of naked and coated CeO_2 nanoparticles**

Simeon Minić

Department of Biochemistry, Faculty of Chemistry, University of Belgrade

Phycocyanobilin and chromopeptides from C-phycocyanin: structure, biological activity and binding to human serum albumin

Ana Obradović

Department of Biology and Ecology, Faculty of Science, University of Kragujevac

Antiproliferative, antioxidative and promigratory effects of 3-benzyl-5-isoprpyl-5-phenylhydantoin in human breast cancer cell line MDA-MB-231

Ana Obradović

Department for Biology and Ecology, Faculty of Science, University of Kragujevac

Cytotoxic and antioxidative effects of Oligo-Grapes product and ethanolic extracts of pomace and red wine on HCT-116 and SW 480 cell lines

Milica G. Paunović

Department of Biology and Ecology, Faculty of Science, University of Kragujevac

Fenitrothion-induced changes of haemato-biochemical and oxidative stress parameters in rat blood: Protective role of selenium and vitamin C

Diandra Pintać

Department of Chemistry, Biochemistry and Environmental Protection, Faculty of Sciences, University of Novi Sad

Phenolic profile and antioxidant potential of grape vine leaves

Vid Puž

Department of Biochemistry, Faculty of Chemistry and Chemical Technology, University of Ljubljana

Structural insight into the myotilin-actin interaction

Slavica Ranković

Centre of Research Excellence in Nutrition and Metabolism, Institute for Medical Research, University of Belgrade

Liver phospholipid fatty acids composition in response to different types of diets in rats of both sexes

Ana Sedlarević

Department of Life Sciences, Institute for Multidisciplinary Research, University of Belgrade

Gall formation by *Rhinusa pilosa* alters the carbohydrate composition in *Linaria vulgaris* stem

Marinela Šokarda Slavić

Centre of Chemistry, Institute of Chemistry, Technology and Metallurgy, University of Belgrade

Rational design of raw starch degrading α-amylase from *Bacillus licheniformis* 9945a for possible surface binding sites identification

Milena Vukić

Department of Chemistry, Faculty of Science, University of Kragujevac

***Eryngium serbicum* as new source of cytotoxic and antibacterial agents**

Foreword

Dear Colleagues

It is my distinct pleasure to welcome you to the 6th Conference of the Serbian Biochemical Society, entitled "Biochemistry and Interdisciplinarity: Transcending the Limits of Field". It is an honor for me to be selected as the Editor of Proceedings of the Conference. I am grateful to the Steering Committee of Serbian Biochemical Society for giving me this opportunity to shape the premiere forum in biochemistry in the region. We have been tremendously fortunate to have Mihajlo B. Spasić as the first Editor. He nurtured this Conference (and Society) through its re-starting years as it grew in quality and relevance. Clearly, following in his footsteps is a challenge.

We have invited Djuro Josić from the University of Rijeka and eight experts from four major universities in Serbia to give lectures at the 6th Conference. The visit of our dear colleague from Croatia is a part of an initiative for closer collaboration within FEBS3+ (Croatia, Hungary, Slovenia, and Serbia) Meeting Programme that was established by FEBS in 2010. We have also invited students at the final years of PhD studies to present their work in our Proceedings as Abstracts. Official languages at the Conference will be Serbian, Croatian, and English.

I would like to express my gratitude to the members of the Scientific Board who suggested lecturers and to all respected colleagues who accepted the invitation.

Editor of the Proceedings
Ivan Spasojević

Proceedings

Proteomics and glycomics as a tool to follow cell injury and malignant modification

Djuro Josić[1,2*], Martina Šrajer Gajdošik[2,3], Uroš Andjelković[1,4], Lucas Breen[2]

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The critical molecular and cellular mechanisms involved in development, progression and metastasis of tumours remain elusive. We demonstrated on rat models that normal prostate epithelial cells undergo spontaneous transformation after more than 85 repeating passage steps ("high passage"). This effect can be observed by the acquisition of anchorage-independent growth and tumourigenicity of the cell when injected into immunodefficient mice. Minor subpopulation of these cells (SAI) had the ability to migrate through soft agar and they also showed marked differences in morphology, proliferation, motility and expression of signalling proteins as well as higher tumourigenic potential. In addition, the changes of extracellular vesicles (EV) of rat liver before and after injury were presented. Extracellular vesicles from stem-like liver epithelial cell line WB-F344, their chemically transformed lines GP7TB and GP7TB.SAI, a soft agar invasive population, were investigated. Apart from changes in number, size and proteomic content, the GP7TB.SAI-derived EVs significantly simulated NK-mediated cytotoxicity, opposite to those purified from the less malignant GP7TB line. Furthermore, we investigated changes in IgG and IgM antibody glycosylation patterns in patients undergoing image guided tumour ablation. Although the glycosylation of antibodies in patients was found to vary with cancer type, discernable patterns of glycosylation change of both antibodies based on successful treatment of tumours by ablation were not identified. These findings suggest that glycosylation patterns are indicative of an immune system that is unable to prevent different types of cancer, rather than products of immunostimulatory response to the ablation and destruction of tumour itself. Present strategy opens a way for parallel determination of proteomic and glycomic changes by use of high-resolution, high throughput methods, and their future use for detection of new biomarker for disease diagnosis and prognosis.

Differentiation *vs.* apoptosis – new concept in the treatment of aggressive cancers

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Since the discovery of the first chemotherapeutic agent, the main concept of aggressive malignant disease treatment is established on the idea how to kill transformed cells. To overcome resistance to cell death inducers, protocols proposing a treatment based on the combination of few agents with different way of action are created. The outcome of this approach is amplified healthy tissue intoxication but, unfortunately, not efficacy. Why the agents effective "*in vitro*" mostly become inactive "*in vivo*" or inefficient in clinical trials? Can we limit the tumour growth without killing the cancer cells? During malignant transformation, cells enter the process of dedifferentiation passing through different stages in opposite direction of their embryonal development and maturation. Poor cellular differentiation is, without doubt, in correlation with tumour aggressiveness and resistance to chemotherapy. Rarely mentioned role of apoptosis in initiation of surrounding low-differentiated cells division, as a main mechanism of tissue regeneration or wound healing, could be also responsible for questionable efficiency of cytotoxic therapies in aggressive types of cancers. Desirable strategy that enables an evasion of compensatory proliferation and tumour repopulation, should be to stimulate cell maturation towards terminally differentiated phenotype or establishment of the senescent phenotype. Both processes are related to the loss of proliferative potential and characteristics of the "stemness". These will allow the elimination of any signal for further tissue renewal, such as compensatory proliferation, and tumour mass will be tranquilize and slowly returned under control of the organism to which it belongs. Beside physiological molecules competent to trigger the differentiation of pluripotent cells, powerful compounds able to promote this kind of changes are mainly derived from nature. Previously, we reported that numerous herbal and synthetic constituents endorse differentiation of extremely aggressive cancers such as glioblastoma or melanoma towards normal primary phenotype or trans-differentiation towards distant tissue cells phenotype developed from the same embryonal origin.

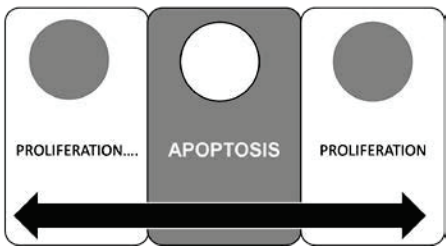
Introduction

Even after decades of research and extraordinary costs, cancer remains the one of the major health problem, with enormous ability to evade antitumour immune response as well as treatments applied. Along all these years of intensive research, scientist all over the world are trying to create agents able to selectively eliminate malignant cells primarily through induction of cell death [1,2]. Despite numerous contradictions raised from the results published on this subject in the era of modern science, the promotion of programmed cell death – apoptosis is recognized as preferential virtue that experimental therapeutic needs to have to be qualified as antitumour compound. Than it might be extremely unexpected that high rate of apoptosis in tumour tissue are linked to poor prognosis for cancer patients [3]. Without taking into account this observation, induction of apoptosis has still in use as a main criterion for the selection of potential therapeutics from the *in vitro* investigation through animal models for clinical trials. Unfortunately, even with impressive methodology and technical support, this concept did not bring expected benefit to medical science but rather generated confusion. After more than a half of century, one of the first created chemotherapeutics – cisplatin and its derivatives are still in the group of the leading drugs in therapy of metastatic malignancies [4]. The effects of cisplatin are highly limited by its toxicity and resistance development. Besides, analysis performed on cell lines *in vitro*, offered additional explanation what could be behind limited efficacy of this and similar therapeutics, but also brought a new insight in our understanding of cancer as a disease. The phenomenon experienced by the most of the researchers in experimental oncology is that the potential of tested compounds is not just defined by the type of the cell, but also is affected by the density of the culture [5]. Paradoxically, confluent cell culture becomes almost resistant or even proliferates in response to the treatment with doses highly cytotoxic for the same cells seeded in medium or low density. This very simple experiment revealed important fact – cell behaviour, independently from the intracellular signature specific for the certain type of the cell, becomes extraordinary different when they are in intimae communication.

Apoptosis as a trigger of tumour repopulation

One of the major omissions in oncology research is neglecting the significance of inter-cellular communication between transformed cells, especially in the context of apoptotic cell death presence. Outcome of apoptosis is entirely different when it is analyzed in the context of intercellular communication [6]. Namely, apoptosis is considered as a silent cell death. Apoptotic cells orchestrate their own "funeral" by delivering many well-synchronized signals that could be classified as "find me", "eat me" and "keep out" [6,7]. Releasing numerous mediators, apoptotic cells arrange phagocytes recruitment, effective clearance and the establishment of anti-inflammatory environment, respectively [8-11]. However, this type of programmed cell death has a profound non-cell autonomous effect on the neighbourhood, affecting cell division, fate and remodelling [12]. Unexpected roles of apoptosis in tissue remodelling during development, as well as regeneration, became of

essential relevance for appropriate understanding of cancer, seriously jeopardizing actual approaches in cancer therapy. The first evidence that apoptotic cells trigger overgrowths in neighbouring tissues through activation of mitogenic signals came from studies of *Drosophila* wing imaginal discs [13,14]. Impressively, the same phenomenon was found to be crucial for the regeneration of the most primitive multicellular organisms with very flexible forms of intercellular communication and, particularly, functional specialization [15]. Dependent from the context, one of the earliest actions of apoptotic cell is sending the information to surrounding cells about the entering the programmed of cell death [16]. In contact with progenitor cell, apoptotic cell delivers the signal, which triggers the proliferation of neighbours serving as a tissue homeostatic mechanism [16,17]. All these principles could be extrapolated to cancer tissue. Tumour mass is created from numerous cell populations. Bulk cells present majority of transformed cell with exact phenotype signature. Together with them, fibroblast-like subpopulation known as CAF (Cancer Associated Fibroblast) interacts with the bulk cells [18,19]. It was found that these subpopulations present different phenotypes delivered from the same origin, proved by the fact that they carry the same genetic aberrations [20,21]. Very important member of this "society" are stem cells. These cells are silent and low proliferating. However, like in normal cell tissue repopulation, "bulk" cell apoptosis triggers "stem" cell activation and subsequent division. Tumour progression is followed by further dedifferentiation. Highly invasive forms of cancers have been characterized as anaplastic and related malignant cells have been described as low differentiated cells [22]. Moreover, metastatic cells possess all characteristics of pluripotent phenotype [23]. Under these circumstances, induction of apoptosis might be related to extensive division of neighbouring cells (Scheme 1). This process is called compensatory proliferation and is necessary for the repair and regeneration of damaged tissue.



Scheme 1. Apoptotic cell death as a main signal for proliferation and tumour repopulation.

Compensatory proliferation and tumour progression

Evolutionary, compensatory proliferation is described for the first time in primitive metazoa explaining their impressive regenerative potential. Chera et al. found that when the hydra body is cut transversally in half the lower part produces a new head by apoptotic cell acting [15]. Namely, dying cells present in this part become a source of Wnt ligands

enabling the proliferation of neighbouring tissues. The net effect of these events is regeneration of the missing head. Similar mechanism was observed in vertebrates during wound healing and liver regeneration, where the signals delivered from damaged hepatocytes conduct the regeneration of missing part [24,25]. However, in the case of massive damage like in chronic injury, bone marrow resident stem cells are important source for liver epithelial cells replacement [26]. Interestingly, Davies and Lineweaver, astrobiologists, hypothesized that cancer is an atavistic condition, developed from the genetic or epigenetic malfunction, which re-established ancient genes, essential for cellular cooperation in primitive multicellular form of life [27]. Affected tissue become autonomous and represents some kind of phylogenetic regression. Raising evidence about cancer progression after applied chemo or radiotherapy especially in advanced cancers suggested that compensatory proliferation could be responsible for low curing rate of conventional therapeutic approaches.

It was found that key executioner caspase necessary for completing of apoptosis, caspase-3, is also critical for translation of proliferative signal (Scheme 1) [3]. Rapid proliferation of the surviving cancer cells that repopulate the tumour was observed after radiotherapy of patients. Huang et al. found positive correlation between high expression of active caspase-3 in head and neck carcinoma and advanced breast cancer and poorer prognosis [3]. This finding opposes to general expectation that caspase-3 deficient cells would be resistant to apoptosis and therefore unresponsive to therapy based on induction of apoptotic cell death. It has been reported by Kim and Moretti that caspase inhibition resulted in radio sensitization of lung and breast cancers *in vivo* [28]. It is indicative that caspase-3 deficiency, as a good prognostic marker in advanced malignancies, could be related to phenomenon of compensatory proliferation. The mechanism of this repopulation, like in tissue regeneration, involved prostaglandin E2 and its ligation to appropriate receptor expressed on progenitor cells [3,29]. Paradoxically, it seems that the induction of cell death by the therapy in metastatic malignity, is at the same time a major limit for its efficacy. This undesirable effect of aggressive treatments in cancer disease is not just limited to the events in tumour mass. Long distance activities that organism arrange in response to "tissue" injury under different physiologic conditions such as inflammation, tissue repair are important also for neoplasia [6]. Recent studies demonstrate that a variety of mesenchymal stem cells from the bone marrow are recruited at injury sites in the manner described above in liver regeneration. Kerbel found recruitment of bone marrow derived stem cells in tumour soon after application of maximal tolerated dose of cytotoxic chemotherapeutics, such as paclitaxel or cyclophosphamide, or microtubule inhibiting 'vascular disrupting agents' [30].

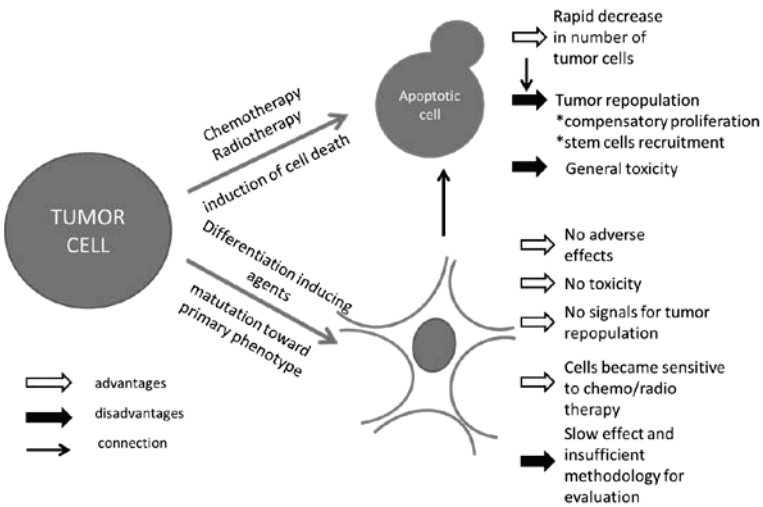
According to this, actual understanding of cancer, as well as concept of cancer treatment, has to be seriously redefined. Strategies which take into account compensatory proliferation and tumour tissue repopulation events in response to aggressive induction of tumour cell death by therapeutics are based on malignant cell plasticity and its potential to overpass different maturation stages [31]. Interplay between phenotypically diverse subpopulation dynamically occurs in tumour mass and microenvironment in response to a

wide range of stimuli. Cytokine, chemokine and other molecules crucial for intercellular communication, migration and signal delivering, possesses differentiation-inducing properties [32,33]. In parallel, numerous mediators from the same panel produced by the neoplastic cells, stromal and recruited immune cells, trigger heterogeneous signals able to promote phenotypic transformation of cancer cells. During disease progression, tumour entity conducts all activities toward invasion and dissemination. General tendency of these events is further cellular dedifferentiation. One of the best explored processes that illustrate this is the phenomenon of epithelial mesenchymal transition (EMT). Like many other processes described in tumour microenvironment, EMT is important for numerous developmental stages, as well as wound healing and organ fibrosis. In EMT of neoplastic cells, epithelial cells lose their cell polarity and cell-cell adhesion, and gain migratory and invasive properties to become mesenchymal stem cells [34]. These are multipotent stromal cells that can differentiate into a variety of cell types. Once that colonize appropriate site, tumour cells re-express E-cadherin and other epithelial markers and this process is described as "mesenchymal-to-epithelial transition" [35]. However, those low differentiated re-established epithelial cells, in contact with new microenvironment, passing through novel transformation regarding to their enormous plasticity [36]. Hypothetically, according to previous findings, progenitor features of metastatic cell subpopulation create the environment in which the apoptosis is far from being silent. Compensatory proliferation and bone marrow stem cells colonization could be completely responsible for tumour expansion after injury triggered by hypoxia or inflammation or more aggressively by some chemotherapeutic or radiotherapy. Theoretically, apart from undesirable compensatory proliferation, progenitor clones could be influenced by various molecules produced by the neighbouring, immune or stromal cells, as mentioned above. Some of these mediators can triggered the maturation of pluripotent cells and possibly limit the expansion of metastatic clones. In parallel, apoptotic cells precedes the mitogen signal to stem but not to cells committed towards a differentiated phenotype that is more susceptible to cell death inducers.

Differentiation-induced therapy in cancer treatments

Differentiation-induced therapy implicates the utilization of agents with the ability to induce maturation process in cancer cells promoting phenotypic change so that they resemble normal healthy cells and making them more vulnerable to therapeutics (Scheme 2). One of the differentiation-based therapies that are now in the clinical practice bringing significant improvement to the patients and transforming the management of in the treatment of acute promyelocytic leukemia (APML) is the addition of retinoids therapeutic approaches [37]. In the 80's, Huang et al. published that treatment of APML patients, mostly non-responsive or resistant to previous chemotherapy, with all trans-retinoic acid led to complete remission in 95.8% of patients without developing bone marrow hypoplasia [38]. However, the application of differentiation-based therapy for the treatment of solid malignancies still has a lot of boundaries. Better insights into the biology of tumour cell, identification of signalling pathways responsible for the re-activation of terminal

differentiation as well as understanding of consequences of applied conventional therapy would certainly be helpful in the development of novel strategies based on phenotypical transformation of malignant cells. Plenty of literature data confirmed that cell lines derived from solid tumours are accessible to differentiation by diverse inducers *in vitro*. The list includes: neuroblastoma [39], glioma [40], breast cancer, colon cancer, pheochromocytoma [41], retinoblastoma [42], melanoma [43], pancreatic cancer [44], colon cancer [45-47], lung cancer [48,49], and liposarcoma [50]. The list of potential inducers is even longer but can be classified as cell- and tissue-type specific, like for example, nerve growth factor for neuronal differentiation of neuroblastoma cells, or nonspecific agents like ATRA, dimethyl sulfoxide, active form vitamin D_3, histone deacetylase inhibitors, peroxisome proliferator-activated receptor-γ (PPARγ) agonists, etc [51]. Also, both naturally occurring compounds, as well as synthetic drugs, are able to trigger the differentiation of malignant cells [52,53]. Interestingly, it was observed that even drugs approved for other medicinal application can be useful as differentiation inducers in the treatment of malignant cells.

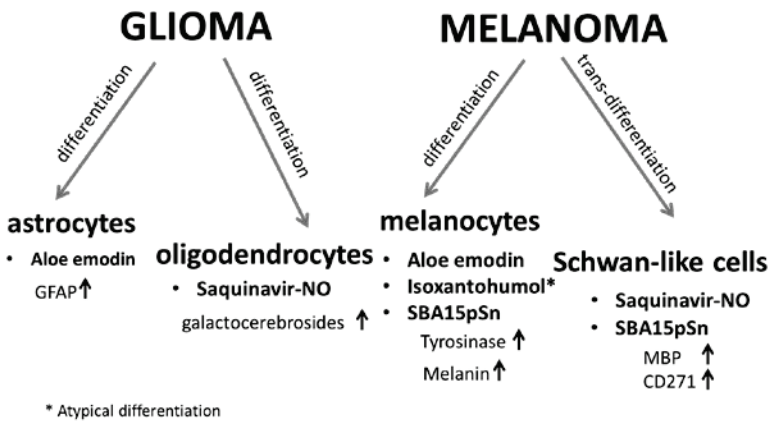


Scheme 2. Induction of cell death *vs.* induction of differentiation in cancer treatment.

Glioma cell differentiation

Glioma is a general term used to describe different types of glial tumours: astrocytoma, oligodendroglioma, and glioblastoma. More than 60% of tumours in the CNS belong to this group [54,55]. Despite major improvements in the understanding of the biology of gliomas as well as gliomagenesis, therapeutic progresses in the past decades remain symbolic. Existence of cancer stem cells with a potential to recreate the original tumour in full heterogeneity, explain the high rate of recurrence after the applied therapy in

glioblastoma multiforme patients. Compensatory proliferation of pluripotent malignant cells in response to death triggered by the chemo or radiotherapy, possibly lead to immediate or delayed tumour progression which is often described as resistance to the therapy. Differentiation of pluripotent cell subpopulation should limit repopulation triggered by toxic therapeutics. In addition to this, more mature phenotype display better response to conventional therapeutics [56]. Differentiation, proliferation and tumour cell death are regulated by the same signalling pathways and cell decision to go toward one of the mentioned directions is a net effect of the interplay of numerous signals. Therefore, it was shown that cAMP/PKA is involved in growth, differentiation and apoptosis at protein and gene level [57]. cAMP elevating agents are able to promote differentiation of gliomas [58,59]. For example forskolin, diterpene extracted from the roots of the *Coleus forskohlii* that activates adenylate cyclase and catalyzes cAMP synthesis, induced differentiation of somatic cell hybrid NG108-15 neuroblastoma/glioma cells [60-62]. Further, cholera toxin, major virulent factor of *Vibrio cholera*, catalyzed ADP-ribosylation of Gs protein and subsequently upregulated cellular cAMP [63]. In C6 cells, forskolin upregulated GFAP through low cyclin D1 maintenance [64]. In PC12 and neuroblastoma cells, β subunit of cholera toxin reacts with ganglioside GM1 and promotes neuronal like differentiation [65]. Maturation of glioma cell lines triggered by cholera toxin was followed by GFAP upregulation and decreased amount of Ki-67 [66]. While in this circumstance differentiation was mediated by JAK2/STAT3 signalling cascade, some other agents isolated from natural sources, as well as synthesized compounds, engaged other signalling pathways such as MEK/Erk or PI3K/Akt axis [53,67]. Plasticity of glioma cells raised from their progenitor features. Depending of the stimuli, cells like C6 glioma can differentiate toward astrocytes or oligodendrocytes (Scheme 3).



Scheme 3. Differentiation of cancer cells triggered by experimental therapeutics of natural or synthetic origin.

It has been reported that treatment of C6 cells with herbal compounds, saicosaponins or aloe emodin (AE) induced their commitment to astrocytic lineage [67,68]. Saicosaponins are triterpenoid compounds isolated from *Bupleurum* species. AE is an herbal antraquinone isolated from roots, barks and leaves of numerous plants used in Chinese traditional medicine. Morphological transformation under AE treatment was followed by increased expression of GFAP caused by inhibition of ERK1/2. Moreover, it was possible to mimic this effect by PD98059, specific ERK inhibitor, confirming once more the relevance of MAP kinase signalling pathway in differentiation of C6 glioma. On the other hand, treatment of C6 cells with HIV protease inhibitor saquinavir modified by covalent attachment of nitric oxide moiety (Saq-NO) forced them to oligodendrocytic lineage (Scheme 3) [53]. Even after short exposure to Saq-NO cells permanently obtained low proliferating phenotype with up-regulated expression of membrane galactocerebroside as a marker of mature oligodendrocytes. Differentiation of glioma cells in this circumstance was mediated by up-regulated expression of p53 and concordant with previously described role of this molecule in neuronal tissue cell maturation.

Melanoma cell differentiation

The incidence of melanoma in Europe is rapidly growing and is generally higher in women than in men. Also, the highest incidence was noted in Northern and North-Western countries. While primary melanoma are curable by surgical excision, metastatic melanoma is almost resistant to conventional therapy [69-71]. Negligible benefit of currently available treatments and high general toxicity indicates the need for novel strategies that are able to overcome chemoresistance. A promising approach involves the application of agents that can promote the maturation of pluripotent subpopulation in primary tumour as well as metastatic foci. Differentiation-based therapy was created on the assumption that specific melanoma cells exhibited abnormal pattern of differentiation opening the possibility that through reprogramming of cancer cells these agents are able to abrogate the proliferative rate and promote the maturation toward adult phenotype. Experiments done at the end of last century revealed that treatment of human melanoma with INF-β and antileukemic compound mezerein triggered the maturation of melanoma phenotype expressed through altered morphology, different panel of antigens and upregulated melanogenesis [72]. Unfortunately, differentiation triggered by many experimental agents resulted in just transitory change of melanoma phenotype conditioned by the presence of the therapeutic. Various agents, either physiological or pharmacological, possess the potential to reprogram malignant cells. Some of them are naturally occurring compounds such as saicosaponins, anthraquinone, and flavonoids, while the others are synthetic and even chemotherapeutic drugs [52,73,74]. We and others revealed that mouse melanoma B16 cell line is able to transform to phenotype resembling primary melanocyte. As differentiation-inducing agents we used different compounds isolated from plants like AE or isoxanthohumol (IXN) (Scheme 3) [52,75]. Exposure of cells to AE was followed with the loss of proliferative properties accompanied with the appearances of dendritic-like prolongation. Biochemically, increased activity of tyrosinase, an enzyme crucial for melanin synthesis,

as well as melanin quantity was elevated in comparison to untreated control (Scheme 3). At intracellular level, cells exposed to AE increased p53, cyclin D and ERK1/2 expression. These cells, after short pulse with experimental substance *in vitro*, were not able to induce tumours in syngeneic strain of C57BL6 mice, confirming once more that melanoma cells differentiate into non-malignant counterparts [52]. Similarly, Tabalocci et al. showed that transformation of metastatic clone of B16-F10 cells upon AE correlated with a significant increase of the activity of the transamidating form of TG2, and cell adhesion and aggregation [76]. While in these circumstances, pattern of differentiation was completed and resulting phenotype resemble primary melanocyte, same cell line exposed to IXN underwent to atypical transformation manifested through slow-down of proliferation, enhanced tyrosinase activity but not melanin content (Scheme 3) [75]. Differentiation upon IXN treatment is probably due to inhibition of p70 S6 kinase and its target, S6 protein activity. p70 S6 kinase controls all many processes related to mentioned phenomenon like protein synthesis, cytoskeletal rearrangement, cell survival and proliferation. The other possible mediator of differentiation process can be elevated p38 MAPK [75]. In the early 90's, it was shown that cytostatic drugs alone or in combination with other compounds are also able to induce the differentiation of B16 cells [77]. This effect is limited to subtoxic dose range or can be related to cell cycle phase or even intracellular communication. Accordingly, we recently published that application of metal based drug organotin (IV)-loaded mesoporous silica nanomaterials (MSN) triggered differentiation of B16 melanoma with the appearances of melanosomes and biochemical markers of senescent cells (Scheme 3) [78]. Observed maturation process was followed by strong activation of p38 underlining the relevance of this protein for transformation of melanoma cells. The usage of nanomaterials as a delivery systems enables the application of subtoxic doses of cytostatic drugs, which efficiently suppressed tumour growth. Interestingly, it was described that some compounds promoted change of melanoma cells' morphology but without up-regulated expression of melanocytic markers. This process is called trans-differentiation and has been considered as a phenomenon where tumour cells change their fate and differentiate into other cell types [79]. Melanoma cells display plasticity to differentiate along the neural and endothelial pathways [80]. The expression of neural specific proteins in melanoma has been described long ago [81-83]. This is connected with the same embryonic origin. We previously described that HIV protease inhibitor Saq-NO led to mild increase of myelin based protein expression together with a diminished tyrosinase activity but no change in the quantity of melanin in B16 melanoma cells. These data indicated that cells exposed to Saq-NO adopted a Schwann-like phenotype, which represents the end stage of involution of normal melanocytes [53]. Similarly, A375 human melanoma cells that are known as highly aggressive, metastatic and chemotherapy resistant cell line, can enter trans-differentiation upon the treatment with mentioned organotin-loaded MSN (in press). This cell line constitutively expresses inducible form of NO synthase and therefore, produces NO which is endogenous factor for their growth. A375 is amelanotic melanoma and thus is considered as low differentiated resembling melanoma stem cells [84,85]. Treatment with organotin-loaded MSN led to the appearance of elongated morphology of cells, development of senescence, raised expression of CD271, low-affinity nerve growth

factor receptor, known as an early stage marker of Schwann-like cells confirming the trans-differentiation process (in press). On the other hand, the treatment down-regulated the expression of to Wnt/β catenin, Notch 1 and Oct3/4, known mediators of the pluripotency and invasive phenotype maintenance.

Clinical observation of differentiation based therapy

Clinical evidences about the therapeutic effect of differentiation-based therapy on solid malignancies are still poor. One of the earliest evidence is the induction of differentiation by PPARγ ligand, troglitazone, in patients with liposarcoma. In three patients with intermediate to high grade liposarcoma, tumour biopsies revealed histological and biochemical differentiation followed with extensive lipid accumulation in tumour cells and decreased expression of Ki-67. According to these parameters triglitazone triggered the lineage-appropriate differentiation of liposarcoma cells [50]. Further, Grosso et al. showed that trabectedin, antitumour drug isolated from the sea squirt, in 17 of 23 patients with advanced myxoid liposarcomas promoted tissue density changes which precede tumour shrinkage. In responsive patients long lasting tumour response was observed [86]. Studies evaluated the effect of depsipeptide, histondiacetilase inhibitor, in patients with progressive recurrent and/or metastatic non-medullary radioactive iodine (RAI)-refractory thyroid cancer. In two patients they found restoration of RAI avidity confirming the differentiation of thyroid tumour cells [87].

Conclusion and future direction

While until today differentiation-based therapy finds its place in the treatment of haematological malignancy only, some of the pioneers works indicated that it should have perspective in certain type of solid tumours. This approach requires prolong period of observation and lacks sufficient, exact parameters to confirm its relevance in clinical practice. On the other hand, a plenty of evidence from preclinical studies *in vitro*, *in vivo* as well as on human samples obtain from patients, testify it enormous potential and numerous advantages in comparison to actual approach in advanced cancer treatment (Scheme 2). In the meantime, while we are looking for appropriate experimental setting for this kind of therapy, combination of differentiation-inducing agents with conventional chemotherapy or radiation therapy might be valuable. Moreover, evaluation of molecular mechanisms related to tumour differentiation therapy might offer novel molecular targets. Finally, definition of concrete defect at molecular level responsible for the development of pluripotent phenotype in invasive form of cancers could serve as a base for creation of highly specific drugs, able to reverse cancer cells toward functionally-committed stage.

Acknowledgments

The work was supported by Ministry of Education, Science and Technological Development of the Republic of Serbia (Grant No 173013).

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Dopamine and adenosine G protein-coupled receptor heteromers as pharmacological targets in neuroendocrine lung carcinoids

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Neuroendocrine tumours of the lung form a heterogeneous group of tumours characterized by neuroendocrine morphology and expression of neuroendocrine markers. Typical and atypical carcinoids usually occur in never-smokers and are mostly centrally located in the lungs. Although their incidence is relatively low (up to 2% of all pulmonary neoplasms), it has dramatically increased in the last 30 years. Carcinoids have a fairly favorable prognosis, with a 5-year overall survival of around 60–90%, but are therapeutically challenging because of frequent systemic metastases decades after diagnosis. There has been considerable interest in novel effects of adenosine and dopamine receptor activation in oncology, in particular in the control of cell growth and differentiation. It has previously been shown that dopamine D_2 receptors form heteromers with adenosine A_{2A} receptor giving rise to a new biological entity with different biochemical and pharmacological characteristics from the monomers. The aim of this project was to evaluate the differences in expression and potential heteromerization of D_2R and $A_{2A}R$ in typical and atypical neuroendocrine carcinoids of the lung, in order to evaluate their potential in neuroendocrine lung cancer therapy.

Introduction

G protein-coupled receptors (GPCRs) are of special interest in oncology as they represent a protein family of receptors with an exceptional chemical diversity of ligands. Moreover, heteromerization is essential for the function of many GPCRs, and the newly formed heteromers have different biochemical and pharmacological characteristics from the monomers [1]. Thus, the anti-cancer targetable pharmacological potential of GPCRs is immense. Targeting the purinergic and dopaminergic signalling pathways has become very popular in molecular oncology, as activation or inhibition of various GPCRs can induce cancer cell death or growth inhibition [2]. Recently, adenosine and dopamine GPCRs have

been recognized as targets for the treatment of various neuroendocrine tumours (NETs) [3,4]. Adenosine A_{2A} and dopamine D_2 GPCRs might be especially interesting as it has been shown that they form functional heteromers with varying characteristics depending on the cell type [2]. This enables the targeting of specific heteromers with known pharmacological characteristics without disrupting the signalling of individual receptors on other cells.

This project was focused on identifying the potential of $A_{2A}R$-D_2R heteromer signalling in neuroendocrine lung cancer therapy. Lung NETs represent a heterogeneous population of tumours, ranging from well-differentiated bronchial carcinoids to highly malignant and poorly differentiated small cell lung cancer and large cell neuroendocrine carcinoma [5]. They are all characterized by neuroendocrine morphology and expression of neuroendocrine markers. Although the incidence of typical (TC) and atypical (AC) carcinoids is relatively low (1–2% all pulmonary neoplasms), their occurrence has increased over the past 30 years, and they usually occur in never-smokers [6]. When surgical resection is not an option, they are difficult to treat because of their insensitivity to both radiation and chemotherapy. Also, frequent recurrence or systemic metastasis can occur decades after the resection of the primary tumour [7]. Very little is known about the potential prognostic and/or predictive biomarkers of lung NETs, although there is a constant progress in the field.

The aim of the first phase of this project was to detect the genetic and protein expression and heteromerization of adenosine A_{2A} and dopamine D_2 receptors in neuroendocrine carcinoid samples (good and poor responders to chemotherapy). The second phase will be focused on the assessment of the significance of the heteromers as prognostic and predictive markers for targeted therapy of lung NETs.

Material and methods

TC and AC patient groups consisted of 26 samples, each group comprising both good and poor responders to standard chemotherapy. The TC patient group consisted of 15 females and 11 males of Caucasian descent, age range 17-75 (median 50), and the AC patient group of 12 females and 14 males of Caucasian descent, age range 29-72 (median 53). Tumour localizations ranged from lower left lobe, upper left lobe, left principal bronchus, upper right lobe, lower right lobe, middle lobe to the lingual trachea. RNA and proteins were isolated from FFPE tumours samples using RNeasy® FFPE kit (Qiagen) and Qproteome® FFPE Tissue kit (Qiagen), and cDNA synthesized from RNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™). Evaluation of genetic and protein expression in TC and AC samples was performed by RT-PCR and Western blot, using GAPDH as a reference. Detection of $A_{2A}R$-D_2R heteromers in tumour samples was performed by Proximity ligation assay (PLA), using the Duolink II in situ PLA detection Kit (OLink, Bioscience). Microscopic observations of heteromerization were made using Olympus FV 300 confocal scanning laser microscope (Leica Lasertechnik, Leica Microsystems). Red fluorescent images were processed with Image J software, and statistical analysis performed using GraphPad Prism V.6, with significance set at $p < 0.05$.

Results

A statistically significant difference in the expression of $A_{2A}R$ and D_2R between TC and AC at the genetic level was not confirmed ($p = 0.6164$ and $p = 0.6914$, respectively) (Figure 1). At the protein level, a statistically significant difference in the expression of D_2R between TC and AC was not confirmed ($p = 0.5464$). However, AC had statistically significant higher expression of $A_{2A}R$ compared to TC ($p = 0.0458$) (Figure 2).

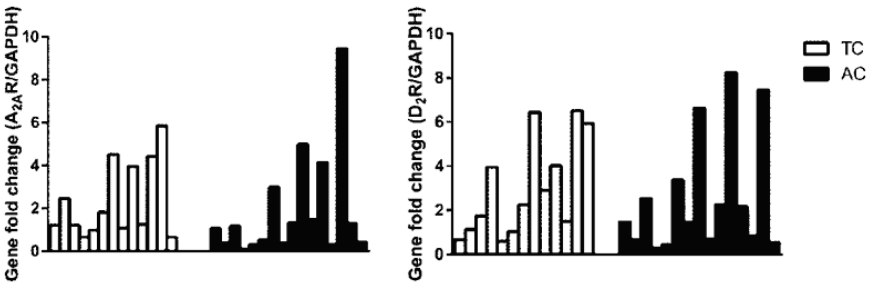

Figure 1. Expression of $A_{2A}R$ (left) and D_2R (right) at the genetic level in TC and AC samples ($p = 0.6164$ and $p = 0.6914$).

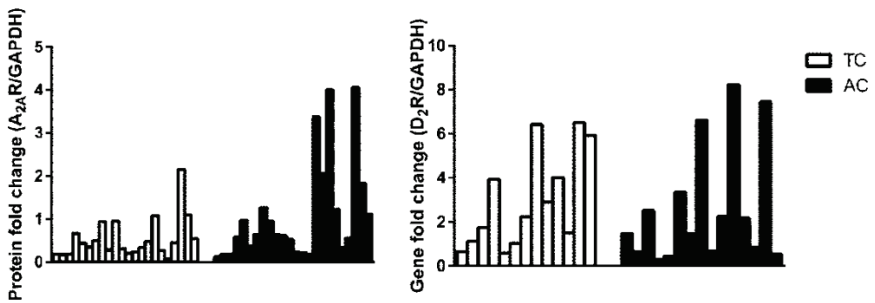

Figure 2. Expression of $A_{2A}R$ (left) and D_2R (right) at the protein level in TC and AC samples ($p = 0.0458$ and $p = 0.5464$).

PLA experiments showed that no $A_{2A}R$-D_2R heteromers were present in TC samples (Figure 3), while 80% of the analysed AC samples showed $A_{2A}R$-D_2R heteromerization (Figure 4).

Conclusions

Typical and atypical neuroendocrine carcinoids showed a significant difference in the expression and heteromerization of $A_{2A}R$ and D_2R. We are currently moving forward with a more detailed pharmacological characterization of these tumour-specific heteromers and correlating them with patient and tumour characteristics and therapy response. This might

discover new targetable biomarkers for neuroendocrine lung carcionoids and enable the development of heteromer-receptor-selective drugs or dual drugs able to interact simultaneously with both units of a tumour-specific receptor heteromer. A new and more effective targeted therapy might be useful for the prevention of the problematic systemic metastasis in neuroendocrine lung tumours.

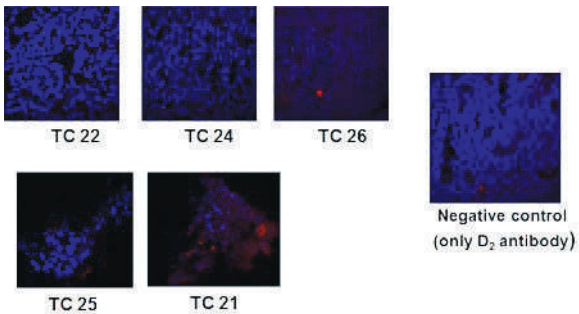

Figure 3. $A_{2A}R$-D_2R heteromers (red) detected by proximity ligation assay in TC samples.

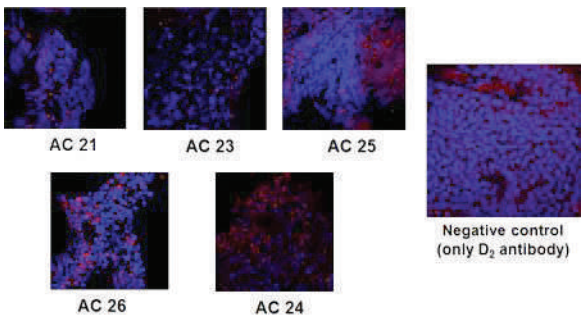

Figure 4. $A_{2A}R$-D_2R heteromers (red) detected by proximity ligation assay in AC samples.

Acknowledgements

This study was supported by a grant from the Ministry of Education and Science of the Republic of Serbia (III41026) and the Boehringer-Ingelheim travel grant (for MC).

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The effects of bioactive compounds on viability of chronic lymphocytic leukemia lymphocytes

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Despite frequent studies, chronic lymphocytic leukemia (CLL) still remains an untreatable disease. Consequently, new bioactive compounds should be considered as valuable clues for a potential future remedy against this malignancy. Therefore, in our investigation we examined the cytotoxicity of eight various bioactive compounds against peripheral blood lymphocytes isolated from CLL patients and peripheral blood mononuclear cells (PBMCs) from healthy individuals *in vitro*. The effects of two types of propolis, chrysin and methanol extracts of *Ligustrum vulgare*, *Teucrium polluim*, *Phellinus linteus* and *Cordyceps sinensis* on viability of CLL cells derived from 28 patients and PBMCs from 16 healthy subjects was determined by MTT assay. The type of cell death induced by chrysin was verified by Annexin V/7AAD assay and acridine orange and ethidium bromide staining assay. Intracellular localisation and endogenic expression of apoptotic proteins including Bax, Bcl-2, cytochrome c and caspase-3 were determined by flow cytometry and fluorescent microscopy. Our results demonstrated that the exposure of CLL lymphocytes to the concentration of chrysin of 10μM and higher, selectively decrease viability of these cells, but had no effect on viability of PBMCs derived from healthy subjects; LC_{50} values of chrysin for CLL cells was 32 μM for 48 h of incubation. Our findings demonstrate that chrysin induced the activation of proapoptotic Bax and a decrease in the expression of antiapoptotic Bcl-2 protein, release of cytochrome c from mitochondria into cytosol and cleavage/activation of caspase-3, subsequently leading to the activation of apoptosis of B-CLL cells. Other studies also delineated the importance of further investigations on other flavonoids such as luteolin, quercetin and resveratrol and their potential in the treatment of CLL. Chrysin selectively induced apoptosis of peripheral blood lymphocytes isolated from human chronic lymphocytic leukemia patients via mitochondrial pathway *in vitro*, so it might have a promising role as a potential future antileukemic remedy.

Introduction

Chronic lymphocytic leukemia (CLL) is a malignant, progressive, clinically heterogeneous disease of hematopoietic tissue that is characterized by clonal proliferation and

accumulation of long-living lymphocytes in the lymph nodes, bone marrow, spleen and other organs [1]. This type of leukemia is a disease of B lymphocytes in the current classification of hematological malignancies by World Health Organization. A disease that was formerly known as T-CLL, is now classified as T-Prolymphocytic leukemia [2]. CLL develops due to oncogenic transformation and clonal expansion of a B lymphocytes subpopulation, which express surface antigens *CD5, CD19* and *CD23*, and have a reduced expression of surface immunoglobulins *IgM, IgD,* and *CD79b* [1,3,4]. These cells can be distinguished by the state of activation, the level of differentiation or cell subset [4].

Epidemiology, pathophysiology and treatment of chronic lymphocytic leukemia

Each year in the United States 15000 new cases of CLL are diagnosed, and about 5000 patients with CLL dies [5]. The average number of patients newly diagnosed with CLL is 4.2 per 100000 persons per year. The disease is more frequent in older people, so that the average number of new patients with CLL is more than 30 per 100,000 persons in the population of people older than 80 years. Median age at patients diagnosed with CLL is 72-years and about 10% of patients are younger than 55 years [6]. CLL is the most common type of leukemia in adults and about 27% of patients with leukemia are diagnosed CLL [5].

The exact reason for the occurrence of CLL is still unknown. CLL is an acquired disorder and familial incidence of the disease is extremely rare [7]. There is no correlation between the occurrence of CLL and action of etiological agents such as ionizing radiation, chemical agents and viral infections [8]. Leukemic cells that progressively accumulate in the blood do not cause the immediate symptoms of the disease. It has previously been considered that CLL arises due to a defective process of programmed cell death in leukemic cells, and not due to disturbances in the proliferation of cells [3]. However, recent discoveries suggested there had been found proliferative niches in the bone marrow and lymph nodes, so CLL was actually a disease caused by the disproportion between the proliferation and apoptosis of CLL lymphocytes [9].

In the past ten years, it has been shown that the CLL was extremely heterogeneous disease [4]. The cells that occur in CLL are “frozen” in the process of differentiation of B cells. However, different genetic mutations occur in the CLL cells of vast majority of patients. A common abnormality is a deletion *13q,* that occurs in more than 50% of patients. In patients who develop disorders associated with *13q14,* usually occurs relatively benign form of the disease that is most commonly manifested as a stable or slowly progressive isolated lymphocytosis [10]. In contrast, the patients who develop trisomy of chromosome 12, have a progressive illness characterized by atypical lymphocyte morphology. This genetic abnormality occurs in about 15% of patients [10].

Deletion of the short arm of chromosome 17 correlated with the rapid progression of the disease, brief remission and generally reduced patient survival. *17p13* deletions are associated with the loss of function of tumor suppressor genes *TP53* and the possibility of changing the cells to enter apoptosis [10]. Changing the function of the gene due to *TP53 17p*

deletions or mutations is associated with decreased survival and poorer response to chemotherapeutics. It should be noted that the part of patients with CLL *TP53* occurring mutations were not accompanied by a deletion *17p13* [11].

It was also discovered that the *BCL2* protooncogene was expressed excessively, and the amount of its antiapoptotic *Bcl-2* protein was increased in CLL cells [12,13]. Interestingly, the genetic abnormalities that were known to cause an increase in the amount of *Bcl-2* protein, such as the translocation of chromosomes 14 and 18, had not been detected in patients suffering CLL. Different studies have shown that increased amount of *Bcl-2* protein was linked to the *13q14* deletion. Two genes marked *miRNA15a* and *miRNA16-1* which were located on *13q14,* encoded a regulatory RNA synthesis marked as micro RNA (*MiRNA).* The transcripts of these genes inhibited the expression of other genes causing mRNA degradation or blocking of transcription [12]. Gene deletions *miRNA15a* and *miRNA16-1* provoked increase of the amount of antiapoptotic protein *Bcl-2* in the cells, due to the loss of micro RNA. Genetic analysis lead to discovery that 70% of patients suffering CLL had a deletion or a mutation in the gene *miRNA15a* and *miRNA16-1,* so it was assumed that this was the reason for the increased amount of *Bcl-2* protein in CLL cells [12,13].

Previously, the treatment of chronic lymphocytic leukemia was based solely on the application of alkylating agents and/or purine analogues. Recently, after the introduction of anti CD-20 antibodies, there has been noticed an increased survival of patients being treated for chronic lymphocytic leukemia (Table 1) [5].

Table 1. Categories of drugs used in the treatment of CLL [5].

Alkylating agents	**Immunotherapy and monoclonal antibodies**
Chlorambucil	Rituximab (anti-CD20)
Bendamustine	Alemtuzumab (anti-CD52)
Cyclophosphamide	Ofatumumab (anti-CD20)
Purine analogs	Lenalidomide (immunomodulatory agent)
Fludarabine (DNA polymerase inhibitor, primase)	
Pentostatin (inhibiting adenosine deaminase)	
Cladribine (inhibiting adenosine deaminase)	**Other drugs**

Programmed cell death

Programmed cell death involves forms of cell death that depend on and are controlled by genetically encoded signal or internal biochemical process of dying cells. Although this term has long been used exclusively for apoptosis, it has been known that programmed cell death includes other types of cell death, such as autophagy and programmed necrosis [14].

Apoptosis or type 1 programmed cell death is a process that was first described by Karr et al. and was characterized by typical morphological and biochemical changes such as cell shrinkage, condensation and fragmentation of nuclei, creating a protrusion on the cell

membrane and separation from neighboring cells or extracellular matrix. Biochemical features include cleavage of nuclear chromatin fragments, ejection of phosphatidylserine to the external environment and specific proteolysis of cellular proteins in the interior of the cells under the action of specific proteases [15].

There are two cellular pathways leading caspase activation and apoptosis: the extrinsic pathway, initiated by "death" receptors and the intrinsic pathway, initiated after cytosolic discharge of mitochondrial derived cytochrome c caused by mitochondrial external membrane permeabilization. Both pathways end with activation of caspase 3 which is executioner caspase. Mitochondrial outer membrane permeabilization by pro-apoptotic protein Bax, is suppressed through the actions of antiapoptotic proteins such as Bcl-2. Therefore, the amounts of Bcl-2 and Bax play a significant role in the execution of apoptosis [16].

Autophagy or type 2 programmed cell death, begins by forming structure consisting of a bilayer membrane surrounding the cytoplasmic macromolecules and organelles destined for recycling. Autophagy plays a key role in cell survival during periods of lack of nutrients and the absence of growth factors [15,17]. In addition to apoptosis and autophagy, there is a cell death called programmed necrosis or type 3 programmed cell death. During this process, main cellular changes leads to swelling of cells, organelles dysfunction and cell lysis [17].

Bioactive compounds

Bioactive compounds are substances that exhibit a specific effect on a living organism, tissue or cell. They may be of vegetable or animal origin or can be artificially synthesized [18]. Natural products are a rich source of compounds which have a wide range of applications in tumor therapy. In addition, a large number of natural ingredients provide basic molecules, which can be modified and used to improve the therapy. Over 70% of the substances used in the treatment of tumors are either substances of natural origin or substances obtained by modifying natural substances [19]. Also, the conjugation of the natural substances with monoclonal antibodies, or with polymers which are used as the carrier molecules, lead to discovery of more effective targeted therapy modalities [18,19]. Since less than 15% of higher plants were systematically examined, investigation of natural substances that could be used as chemotherapeutics deserved increased attention and application of multidisciplinary scientific research [19].

Therapeutic options for patients suffering from chronic lymphocytic leukemia vary widely and depend on the patient's age, various risk factors of the patient and severity of symptoms that the disease causes. A large number of patients live relatively long without therapy, although they had been diagnosed by chronic lymphocytic leukemia. In general, the treatment of complicated disease, and early initiation of therapy has not been proven to be effective in prolonging the life of patients who receive this kind of treatment [1-5,20]. Therefore, and because the treatment itself may cause a number of side effects, it is advisable that the treatment of chronic lymphocytic leukemia does not start until the

disease progresses and some serious symptoms appear. If the doctor estimates that the therapy is required, it is necessary to also consider other factors such as patient's age, general health condition of the patient and the presence of some prognostic factors, such as deletions of chromosome 17 or 11, or a high expression level of *ZAP-70* and *CD38* [20].

A large number of new substances for potential use in the treatment of chronic lymphocytic leukemia are now in the testing stage. Many of these substances target specific parts of cancer cells, while others have a nonspecific mechanism of action. Despite the fact that in recent years there has been progress in the treatment of CLL, common problems were still present. Thus, by applying the ibrutinib, inhibitor which covalently binds to the Bruton's tyrosine kinase, despite the fact that there is a great response in a group of patients with refractory chronic lymphocytic leukemia, 5.3% of patients develop a progression of the disease [20]. Therefore, it is necessary to find agents that will effectively treat chronic lymphocytic leukemia, and with fewer side effects.

Propolis or bee glue, is a natural substance like wax that bees use to seal up cracks or honeycomb. At lower temperatures, propolis is soft and sticky, and at higher temperatures becomes firm and brittle. Its color varies from green to red-brown, depending on the plants that bees use to produce it, and geographical origin. So far, it has been found over 60 species of plants from which bees collect propolis[21]. After some period of time, propolis becomes darker and loses its elasticity on the sunlight. It has a distinctive resinous odor and slight bitter taste [20,21].

Flavonoids include a wide range of plant pigments that can be found in fruits and vegetables. Until now, it has been discovered more than 4000 flavonoids that could be further divided into flavonols, flavones, flavanols, flavanones, anthocyanidins, and isoflavonoids (95-97, 100). Chrysin (5, 7-Dihydroxyflavone) belongs to the group of flavones. All flavonoids have a common chemical structure, and include fused rings *A* and *C* and in the phenyl ring linked to the carbon atom in the 2[nd]-position of a *C* ring (Figure 1). Chrysin differs from other flavones by the presence of hydroxyl groups on the carbon atoms at positions 5 and 7 of *A* ring (Figure 1) [22].

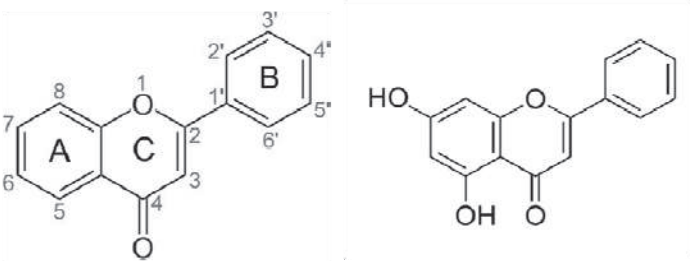

Figure 1. Common chemical structure of flavones (left) and structural formula of chrysin (right) [22].

Chrysin can be found in propolis and plant extracts of *Passiflora caerulea, Passiflora incarnata, Oroxylum indicum, Matricaria chamomilla* and fungus *Pleurotus ostreatus* [22-24]. It was also considered as one of the key molecules responsible for the anticancer properties of propolis [24]. Chrysin exhibited cytotoxic activity and reduced cell proliferation

of murine (B16-F1) and human (A-375) melanoma cell lines through caspase-dependent mechanisms [25,26]. Activation of apoptosis of these cells was accompanied by a redistribution of intracellular proapoptotic protein *Bax* from cytoplasm to the mitochondria [26]. The effect of chrysin on certain leukemic cells was not completely tested. It has been shown that chrysin reduced cell viability and to induce apoptosis in U-937 human histiocytic lymphoma cells, and MO7e acute megakaryocytic leukemia cells. Reducing the viability U-937 cells by chrysin was associated with the activation of caspase-3 [27]. The cytotoxic effect of chrysin was demonstrated *in vitro* on acute monocytic leukemia cells THP-1 and the human promyelocytic leukemia cells HL-60 [28].

Ligustrum vulgare is a bushy, deciduous plant that grows up to 5 meters and it is widespread in Central Europe, Serbia, Crimea, Caucasus, Moldova and Ukraine[29]. It is a common plant in the forest communities of oaks. It is sometimes planted in parks and backyards as a hedge. It is believed that the plant itself and the fruits of plants in particular are toxic, especially for children[30]. The leaves of *Ligustrum vulgare* are spear-like shape, 2-7 cm long and 0.5-2 cm wide, dark green on the face and light green on the reverse side. The flowers are white and form a pyramid-shaped inflorescence. The fruit is a berry, black in color and with a diameter of 0.5-1 cm [29].

Teucrium pollium is shrubby plant named after Teucer, king of Thebes, who recommended to his people the use of this plant for medicinal purposes. In traditional medicine *Teucrium pollium* is used the last 2000 years, and it is widespread in the in mountainous and rocky areas of Middle East and the Mediterranean region. The plant is 40 cm in height, with elongated, oval and slightly serrated leaves. The flowers are white to slightly pink, densely packed on top of the branches [31].

Phellinus linteus is a medical mushroom, which has always been used in the folk medicine of the Far East. The fungus itself grows on willow and elm trees, mulberry, but is rarely found in nature. *Phellinus linteus,* has been used in folk medicine for centuries in the treatment of abdominal pain, inflammation, arthritis, gastrointestinal disorders, diabetes and cancer [32].

Phellinus linteus exhibited inhibitory and proapoptotic effect on certain types of human leukemia cells such as acute promyelocytic leukemia cells NB4, and chronic myelogenous leukemia cells K562 [33].

Cordyceps sinensis is a fungus that parasites on insects, mainly on the head of butterfly larva *Hepialus armoricanus,* although it can be found on other types of butterflies. It has been used for medical purposes in China for over 300 years. *Cordyceps sinensis* contains vast number of bioactive substances such as polysaccharides, nucleosides, cordycepin and other derivatives, mannitol, aminophenol and ergosterol. It has long been used in China and other Asian countries as a dietary supplement and tonic with the ailing, particularly among older patients. It has been proven that chemical compounds isolated from mushroom *Cordyceps sinensis* have nephroprotective, hepatoprotective, antiinflammatory, antioxidant and antiapoptotic effects [34].

Cordyceps sinensis extracts induced apoptosis in human promyelocytic leukemia cells HL-60, by decreasing the cytoplasmatic concentration of antiapoptotic protein Bcl-2*,* inducing

translocation from the cytoplasm to mitochondria of proapoptotic protein Bax, releasing of cytochrome *c* from mitochondria into cytoplasm and activating caspases-2, -3 and -9 [35]. Also, it was shown that the extract of the mushroom *Cordyceps sinensis* inhibited the proliferation of histiocytic lymphoma cells U937 and human promyelocytic leukemia cells HL-60 [36,37].

All these bioactive compounds decreased viability of various cancer cell lines *in vitro*. However, it was unclear how they affected viability of CLL cells.

Preparation of CLL lymphocytes and peripheral blood mononuclear cells

The effects of two types of propolis, chrysin, methanol leaves and fruit extracts of *Ligustrum vulgare*, methanol leaves extract of *Teucrium pollium* and methanol extracts of *Phellinus linteus* and *Cordyceps sinensis* on viability of CLL cells isolated from 28 patients and peripheral blood mononuclear cells (PBMCs) from 16 healthy subjects had been determined by MTT assay. The local Ethics Committee accepted the study and prior to its initiation, the written informed consent was obtained from all subjects according to the Declaration of Helsinki. CLL was diagnosed by establishing the clinical criteria and it was confirmed by immunophenotypic analysis for the expression of CD5, CD19 and monoclonal immunoglobulin in accordance with updated NCI Working Group Guidelines [38]. There were healthy volunteers in the control group without known acute and chronic diseases. Peripheral blood samples from 28 CLL patients that did not receive medications for at least 6 months and 16 healthy control subjects, were included in study. All subjects were non-smokers, without alcohol abuse problems, and none of them performed regular exercise other than daily activities. In addition, subjects did not receive any systemic and topical treatment within 6 months prior to the initiation of the research [39].

All blood samples were obtained in the morning and collected in silicone coated blood collection tubes (Terumo). Peripheral blood samples (9 mL) were centrifuged at 400×g for 10 minutes to separate plasma and cells. Peripheral blood mononuclear and polymorphonuclear cells were separated by single step continuous density-gradient centrifugation with Histopaque 1077. The separated mononuclear cells were washed three times with culture medium RPMI 1640 and resuspended in RPMI 1640 supplemented with 10% autologous serum, 100 IU/ml penicillin G and 100mg/ml streptomycin [39]. The monocytes were removed by adhesion on plastic Petri dishes [40]. Cell number was determined using trypan blue staining [39,40].

CLL cells viability assessment

In order to investigate whether these substances influenced viability of investigated cells, both CLL lymphocytes and mononuclear cells isolated from healthy volunteers were treated with various concentrations of compounds for 48 h and the resulting number of remaining viable cells had been evaluated. The chrysin, leaf and fruit methanol extracts of *Ligustrum vulgare* and a methanol extract of *Teucrium pollium*, reduced the viability of CLL cells, and did not affect the viability of normal mononuclear leukocytes *in vitro*. The

lowest LC_{50} value against CLL cells was demonstrated by chrysin and therefore chrysin was the most effective of all selected bioactive compounds.

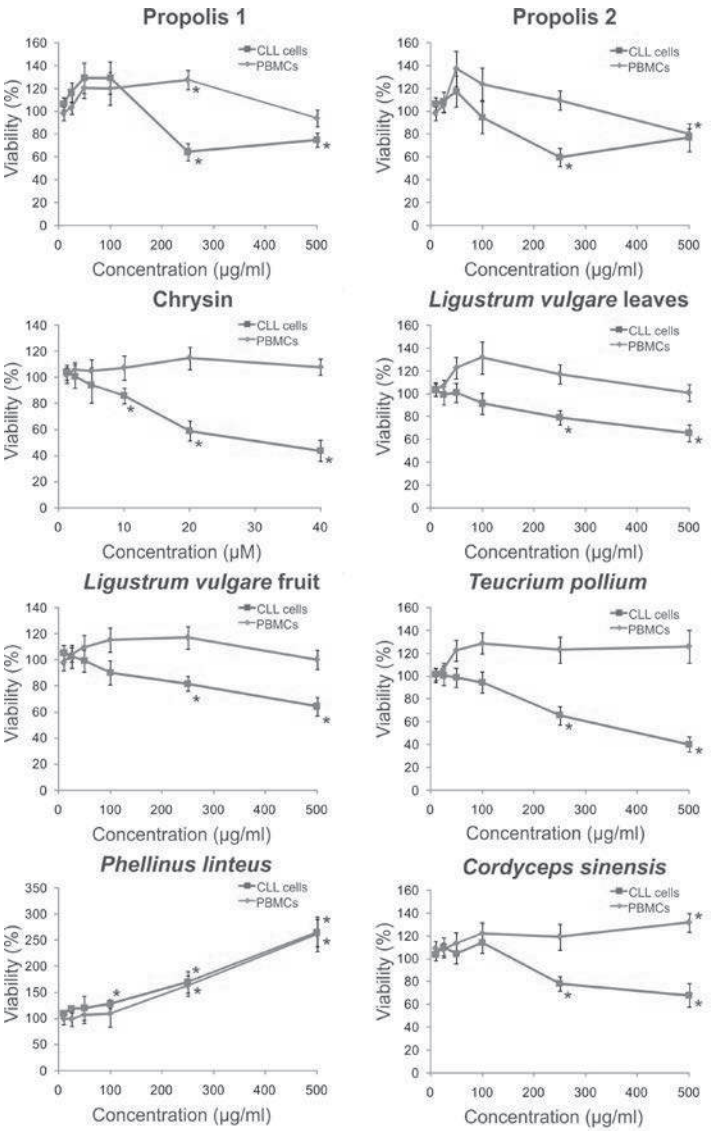

Figure 2. Some bioactive compounds affect viability of chronic lymphocytic leukemia lymphocytes (CLL cells) and peripheral blood mononuclear cells of healthy volunteers (PBMCs) after 48 h of incubation[41].*$p<0.05$ compared to untreated cells.

Quite the opposite, two types of propolis and methanol extract of *Cordyceps sinensis* reduced the viability of leukemic cells, but also affected the viability of normal mononuclear leukocytes (Figure 2). Moreover, methanol extract of *Phellinus linteus* increased the viability of both CLL cells and normal peripheral blood mononuclear cells (Figure 2) [41].

In accordance to MTT assay results, treatment with 20 μM and 40 μM of chrysin significantly reduced the percentage of CLL cells after 48 hours of incubation compared to the viability of healthy PBMC treated with the same concentrations of chrysin. The LC_{50} dose of chrysin for CLL lymphocytes was determined to be 31.91 ± 4.38 μM for 48 hours. On the other hand, exposure of mononuclear cells isolated from healthy subjects to chrysin for 48 hours, had no effect on cells viability at all tested concentrations of chrysin compared to the untreated mononuclear cells ($p > 0.05$; Figure 2) [39].

Afterwards, it was proven that chrysin decreased viability of CLL cells by inducing apoptosis. The type of cell death induced by chrysin was verified by Annexin V/7AAD assay and acridine orange and ethidium bromide (AO/EB) staining assay. Intracellular localisation and endogenic expression of apoptotic proteins including Bax, Bcl-2, cytochrome c and caspase-3 were determined by flow cytometry and fluorescent microscopy. So, it was demonstrated that chrysin selectively induced mitochondrial damage and apoptosis in peripheral blood lymphocytes isolated from human chronic lymphocytic leukemia *in vitro* at relatively low μM concentrations. Although diverse mechanisms of action might contribute to the anti-cancer effects of chrysin, it have been shown that inhibition of Bcl-2 protein expression and activation of Bax were directly involved in chrysin-induced CLL apoptosis. The intracellular disbalance caused by decreased amount of cytosolic anti-apoptotic protein Bcl-2 and increased translocation of pro-apoptotic protein Bax from cytosol to mitochondria, lead to mitochondrial release of cytochrome c and activation of caspase-3. All these cellular mechanisms result by inducing apoptosis of CLL cells [39].

Other studies had previously shown that chrysin reduces cells' viability of various leukemia cell lines such as U937, MO7e, THP-1 and HL-60, but there had not been any data about the effect of chrysin on peripheral blood lymphocytes isolated from human chronic lymphocytic leukemia. It was proven that chrysin selectively decreased viability of leukemia cells and had no effect at mononuclear cells isolated from healthy subjects. Moreover, chrysin displayed the lowest LC_{50} values at CLL cells. Similar results were demonstrated at U937 cell line with $LC_{50} = 16\mu M$ [42].

Chrysin induced apoptosis in peripheral blood lymphocytes isolated from human chronic lymphocytic leukemia that was accompanied by the activation of Bax and decrease of Bcl-2 protein expression, release of cytochrome c from mitochondria into cytosol and cleavage/activation of caspase-3 (Figure 3) [39].

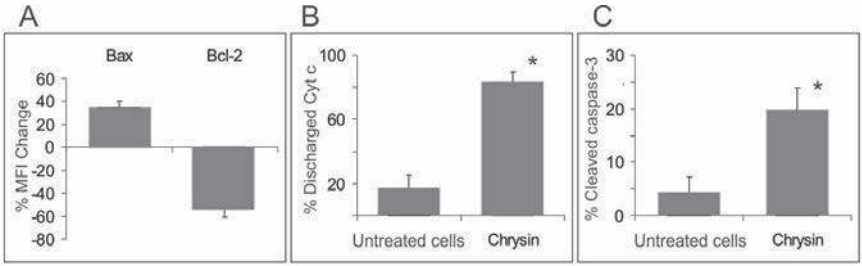

Figure 3. Chrysin increased amount of active Bax and decreased concentration of Bcl-2 in treated CLL lymphocytes, consequently inducing apoptosis by cytochrome c mitochondrial release and activation of caspase-3. CLL cells were cultivated in culture medium alone or with 40 μM chrysin for 24 h and stained with antibodies specific to Bax, Bcl-2, cytochrome c and cleaved caspase-3. Cells were evaluated using flow cytometry. A: Percentage of MFIs suppression or increase compared to untreated cells was determined using formula (Chr-C)×100/C where Chr and C are MFIs of cells treated with chrysin or control cells, in that order. B: Cytochrome c translocation was determined by selective permeabilisation of plasma membrane folowed by flow cytometry. The % of cells with low fluorescence, where cytochrome c was translocated during apoptosis, is displayed. C: The % of cells emiting fluorescence for cleaved caspase-3. *$p < 0.05$ compared to the untreated cells [39].

The most recent study regarding chronic lymphocytic leukemia treatment of Sak et al. provided evidence that other flavonoids such as fisetin, quercetin and especially luteolin also might have important role against chronic lymphocytic leukemia [43]. Luteolin significantly increased the apoptotis of CLL cells by increasing the activities of caspases-3 and -9 and triggering the intrinsic apoptotic pathway.Both fisetin and quercetin had been able to increase the cytotoxic activity of luteolin in CLL cell lines by reducing the IC_{50} values up to four fold. Consequently, luteolin exhibited cytotoxic activity already at low micromolar concentrations that could potentially be used in the treatment of CLL. No other tested flavonoids were capable of sensitizing CLL cells to luteolin pointing to a specific binding of fisetin and quercetin to the targets in the cells which interfere with the signaling pathways induced by luteolin [43].

Also the research conducted by Baran et al., demonstrated the importance of resveratrol and quercetin-induced apoptosis of CLL cells by activation of caspase-3 and cell cycle arrest [44]. Treatment of CLL cells with resveratrol and quercetin induced decreace of CLL cells viability and increased apoptotic cell population through induction of caspase-3 activity. Cell cycle analysis displayed cell cycle arrest mainly in G0/G1 for both compounds. Therefore, resveratrol and quercetin might decrease the viability and reduce the number of CLL cells through induction of apoptosis and cell cycle arrest [44].

Conclusion

Among diverse classes of bioactive compounds present in fruits and vegetables, flavonoids deserve special attention as these plant secondary metabolites can contribute to the prevention and treatment of different malignancies, including chronic lymphocytic leukemia. Although further studies are needed to fully elucidate the mechanism involved

in cell death, chrysin, luteolin, quercetin and resveratrol might have a promising role as future chemotherapeutic agents in the treatment of patients suffering chronic lymphocytic leukemia. Also, these results make the respective combinations of bioactive compounds, especially flavonoids, potentially new possibilities for prevention and adjuvant treatment of CLL by using compounds derived from natural products.

Acknowledgements

This research was conducted as a part of the project of Ministry of Education, Science and Technological Development of the Republic of Serbia, III41010.

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Fatty acids in phospholipids as biomarkers of aging and pathological conditions

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Aging is a natural, complex, and multifactor biologic process. Many of the studies conducted on cultured human cells and animals have revealed that aging is associated with impairment of bioenergetics' functions, increased oxidative stress, attenuated ability to respond to stresses, and increased risk of contracting cancers and age-associated diseases [1]. The free radical theory of aging [2,3], states that age-related degenerative processes are to a large extent the consequence of damage induced by free radicals. Several changes can be observed during aging, which include a reduced capacity to use oxygen along with impaired cardio-circulatory capacity and respiratory adaptation, deterioration of nervous system, and degeneration in muscle mass [4]. Dietary fats in mammals provide essential fatty acids for membrane synthesis, protein modification as well as signaling compounds [5]. High dietary polyunsaturated fatty acids series (n-3 PUFA) increased erythrocyte membrane susceptibility to peroxidation and lipids products in liver or kidney [6], as well as antioxidant enzyme activity in blood [7]. Erythrocytes despite lacking mitochondria are target for free radical exposure, due to the auto-oxidation of hemoglobin under high oxygen pressure. Dietary fats greatly modify erythrocyte membrane composition affecting their susceptibility to oxidation [8]. Fatty acids (FAs) phospholipids in blood or tissue could be biomarkers of pathological conditions or aging process.

Introduction

The phospholipid class, FAs composition and cholesterol content in biomembranes are basic determinants of the physical properties of membranes. They have been shown to influence a wide variety of membrane-dependent functions, such as membrane transport, enzyme activity and receptor function [9,10]. Biological membranes are organized assemblies of lipids and proteins with small amount of carbohydrates. Membrane proteins carry out the dinamic processes associated with membrane [11].

Changes in phospholipid content is a major marker of atherosclerotic changes [12]. Increased intake of the long-chain *n*–3 FAs, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) has been recommended by a variety of regulatory bodies to reduce population risk for coronary artery disease [13]. The FAs profile of the serum phospholipid is related to the

average dietary FAs intake during the last 3 to 6 weeks, while the composition of erythrocyte phospholipids depends on the dietary fat intake during the preceding months [14]. The FAs profile in tissues partly reflects not only the dietary fat intake, but also the efficiency of FAs metabolism in the body [15]. Although mammals are able to synthesize saturated FAs from nonfat precursors and unsaturated fatty acids of the *n*–9 and *n*–7 series, they lack the Δ12 and Δ15 desaturase enzymes (found in most plants) for insertion of a double bond at the *n*–6 or *n*–3 position. Thus mammalian cells cannot synthesize *n*–6 or *n*–3 PUFAs *de novo*. The commonly consumed PUFAs are linoleic acid (LA) (18:2, *n*–6) and α-linolenic acid (ALA; 18:3, *n*–3). Once consumed, these FAs can be converted to longer chain, more unsaturated derivatives [9]. LA is converted *via* γ-linolenic (18:3, *n*–6) and dihomo-γ-linolenic (20:3, *n*–6) acids to arachidonic acid (AA; 20:4, *n*–6). ALA is converted to EPA (20:5, *n*–3) and DPA (22:5, *n*–3). EPA and DHA are termed long chain *n*–3 PUFAs. These FAs are found in oily fish and the preparations known as fish oil [16]. They are involved in regulation of lipid metabolism and processes involved in lipid transport and targeting to tissues [17]. They are components of membranes of all cells, contributing to the fluidity of the membrane and this is believed to play a role in regulation of activity of membrane proteins. Also PUFAs are substrates for the synthesis of bioactive molecules such as prostaglandins (PG), thromboxanes (TX) and leukotrienes (LT) [18].

Eicosanoids formed from the *n*–3 FA are much less potent in causing biological responses, including stimulation of cytokine production and inflammatory reactions than those formed from the *n*–6 series [10]. The immune and inflammatory cells contain many transcription factors including NF kappa B, which regulates the synthesis of cytokines interleukin 1 (IL-1), interleukin 2 (IL2), interleukin 6 (IL-6), tumor necrosis factor- α (TNF-α) and interferon-β. EPA reduces the production of pro-inflammatory IL-1 and IL-6 as well as TNF-α and -β in response to an inflammatory stimulus [19].

When fish oil is provided, EPA is incorporated into cell membrane phospholipids, partly at the expense of AA. There is less AA available for eicosanoid synthesis. EPA inhibits the oxidation of AA by cyclooxygenase. Fish oil decreases production of PG (*e.g.* PGE2), TX (*e.g.* TXA2) and LT (*e.g.* LTB4). PUFAs can potentially reduce platelet aggregation, blood clotting, smooth muscle contraction, and leukocyte chemotaxis, and can modulate inflamatory cytokine production and immune function [17]. Fish oils which are rich in *n*–3 PUFAs have been postulated to be beneficial in several disease states including atherosclerosis, hypertension and arthritis [18,19]. The major *n*–3 FAs found in fish oil are EPA (20:5, *n*–3) and DHA (22:6, *n*–3). Fish oils have been shown to reduce hepatic lipogenesis and VLDL secretion and increase post-heparin plasma LPL activity. Fish oil rich in EPA and DHA inhibits production of PGE2, as well as lymphocyte proliferation and production of interferon-γ. Immune function could be improved by PUFAs, and among them an *n*–3 FAs from fish oil EPA possess the most potent immunomodulatory activities [20]. An increasing number of clinical studies indicate that the consumption of ALA has beneficial effects on human health, particularly on cardiovascular diseases. ALA consumption has been related to protective effects against cardiac death and nonfatal myocardial infarction in prospective and intervention trials [21]. The proportion of saturated

(SFA), monounsaturated (MUFAs), and PUFAs content might have an effect not only on the ALA conversion into EPA and DHA, but also on the concentrations of inflammatory markers and on the plasma lipid profile [22,23].

Materials

The experiments were carried out on young (3 months) and aged (22 months) male Wistar rats, individually housed in stainless steel cages with wired floors, in a room under controlled conditions (12 h light–dark cycles, t = 22 ± 2°C). Forthy Wistar rats were randomly assigned to experimental (n = 10, body weight 283 ± 5 g) or a control group (n = 10, b.w. 278 ± 7 g) for young and experimental (n = 10, b.w. 318 ± 4.05 g) or a control group (n = 10, b.w. 313 ± 6.15 g) for aged rats; thus, the groups were matched in terms of age and body weight. All experiments were carried out according to our Institutional guidelines for animal research and principals of the European Convention for the Protection of Vertebrate Animals Used for Experimental and others (Official Daily N. L 358/1-358/6, 18, December 1986).

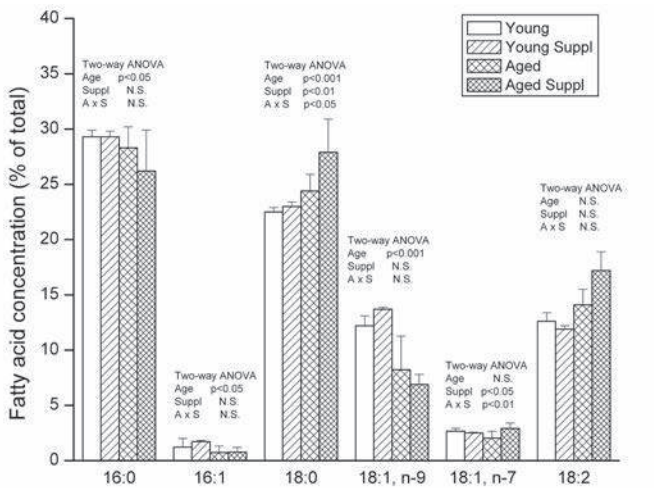

Figure 1. The percentage of palmitic acid, palmitoleic, stearic, vascenic and linoleic acid in plasma phospholipids of young and aged rats supplemented with fish oil. Results were tested by two-way ANOVA with age (A) and supplementation (S) as factors; F values and p significance are presented.

Results

Fish oil supplementation changed fatty acids phospholipids profiles in plasma, especially of longer fatty acids. The supplementation elevated the percentage of stearic acid (18:0), ETA, (20:3), EPA (20:5), DPA (22:5) (Figures 1 and 2), and decreased AA (20:4) (Figure 2). However, the age changes in percentage of palmitic acid (16:0), palmitoleic (16:1),

oleic acid (18:1 (n-9)), linoleic acid (18:2) (Figure 2) and MUFA profiles (Figure 3) were neither reversed nor prevented by fish oil supplementation.

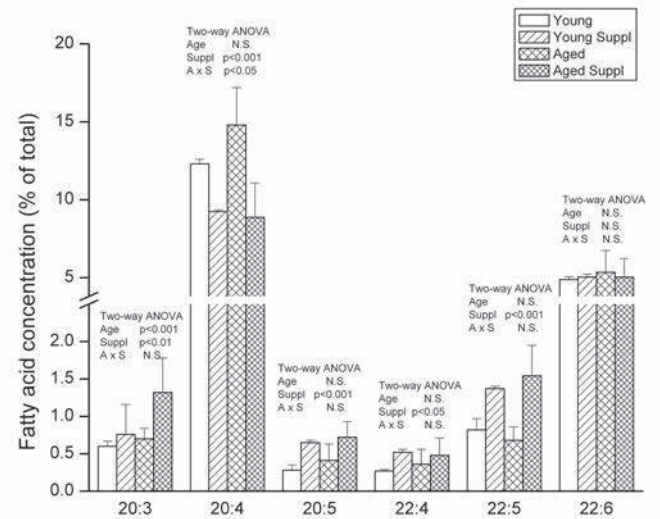

Figure 2. The percentage of ETA, AA, EPA, DTA, DPA and DHA in plasma phospholipids of young and aged rats supplemented with fish oil. Results were tested by two-way ANOVA with age (A) and supplementation (S) as factors; F values and p significance are presented.

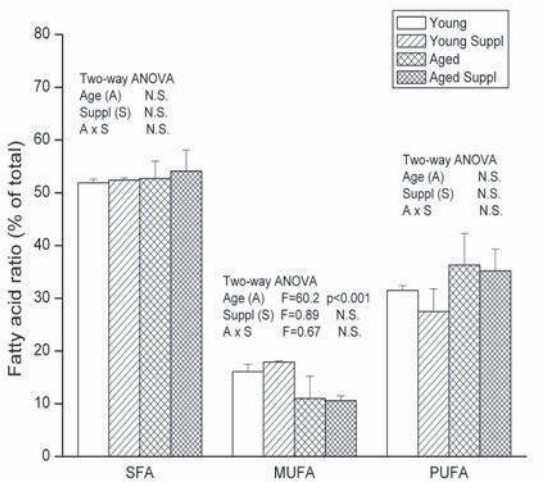

Figure 3. SFA, MUFA and PUFA percentage in plasma phospholipids of young and aged rats supplemented with fish oil. Results were tested by two-way ANOVA with age (A) and supplementation (S) as factors; F values and p significance are presented.

The supplementation elevated the percentage of n-3 fatty acids (Figure 4). It is noteworthy that fish oil supplementation increased percentage of fatty acids such as stearic acid (18:0) and vascenic acid (18:1, n-7) of plasma phospholipids only in aged rats (significant effect of supplementation and interaction $p < 0.05$, Figure 1).

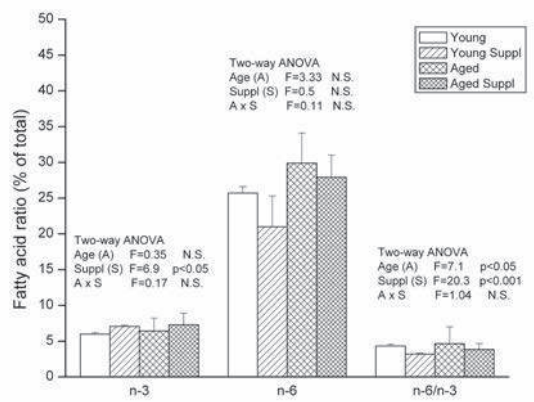

Figure 4. Fatty acids ratio in plasma phospholipids of young and aged rats supplemented with fish oil. Results were tested by two-way ANOVA with age (A) and supplementation (S) as factors; F values and p significance are presented.

Patients with T2DM and fatty acids plasma phospholipids profile changes

In diabetic cells, the amount of glucose oxidized in the Krebs tricarboxylic acid cycle is increased, leading to an increase in generation of reactive oxygen species and resulting oxidative stress plays a key role in the pathogenesis and progression of diabetes and diabetic complications. Fatty acid composition of plasma phospholipids, the FA profile of plasma phospholipids in patients with T2DM (type 2 diabetes mellitus) was significantly different in comparison with control subjects (Table 1). The percentage of total SFA was significantly higher in T2DM patients; however, total MUFA was significantly lower than in control subjects (Table 1). Total PUFA and n-6 PUFA were significantly higher in T2DM patients, particularly LA (18:2 n-6) and arac hedonic acid (AA, 20:4 n-6), whereas n-3 PUFAs, particularly DHA (22:6 n-3), were significantly lower in T2DM patients. The ratio n-6/n-3 PUFA was significantly higher in observed patients as well as the ratio of total PUFA/SFA.

Mechanism of insulin resistance regulation

The mechanism by which n-3 fatty acids may inhibit the production of proinflammatory cytokines has been intensively investigated. FA can bind to the peroxisome proliferator-activated receptors PPARα and PPARγ, which regulate the transcription of target genes,

increasing expression of FA oxidation genes with decrease in hepatic and plasma triglycerides by PPAR a and improving insulin resistance by PPARγ [24]. PPARs can also repress gene transcription by interfering with signaling molecules such as nuclear factor-kB, therefore inhibiting the production of pro-inflammatory cytokines [25,26].

Table 1. Fatty acid profile of plasma phospholipids (% of total fatty acids)

	Control group	**Patients with T2DM**
16:0	26.09 ± 2.10	27.78 ± 2.30
18:0	13.32 ± 2.44	15.91 ± 1.36
16:1, n-7	0.59 ± 0.35	0.63 ± 0.28
18:1, n-9	21.47 ± 2.41	10.47 ± 1.53
18:2, n-6	15.43 ±1.78	23.87 ± 3.02
20:3, n-6	2.74 ± 1.33	0.79 ± 0.22
20:3/18:2 (delta6)	0.13 ± 0.06	0.05 ± 0.01
20:4, n-6	9.47 ± 2.33	11.71 ± 2.84
20:4/20:3 (delta5)	13.0 ± 5.01	4.79 ± 3.24
20:5, n-3	0.79 ±0.54	0.66 ± 0.40
22:4,n-6	0.40 ±0,28	0.68 ±0.32
22:5, n-3	0.75 ±0.68	0.88 ± 0.26
22:6, n-3	2.88 ±1.18	2.59 ± 0.53
20:4, n-6/20:5, n-3	18.92	19.27
Σ n-6	26.10 ± 3,42	39.22 ± 2.66
Σ n-3	3.92 ± 2.07	3.65 ± 0.81
n-6/n-3	8.73 ± 4.17	11.34 ± 3.08
Σ PUFA	30.02 ± 4.64	46.05 ±3.15
MUFA	22.06 ±2.58	12.88 ± 1.68
SFA	39.81 ± 2.42	43.69 ± 2.80
PUFA/SFA	0.76 ± 0.15	1.06 ± 0.14

T2DM, type 2 diabetes mellitus; PUFA, polyunsaturated fatty acids; MUFA, monounsaturated fatty acids; SFA, saturated fatty acids.

Generally, the n-6 PUFA-derived eicosanoids are proinflammatory, and eicosanoids produced from DHA and EPA are less inflammatory [27]. Recent studies have also indicated that metabolic products derived from n-3 FAs such as 17S-hydroxy-DHA, resolvins, and protectins may play a role in the long-term resolution of inflammation. This may attenuate insulin resistance in the context of obesity [28]. Moreover, biosynthesis of AA and DHA requires delta-6 and delta-5 desaturases that are insulin-dependent, and it is possible that their function could be partially depressed in our T2DM, as stated by others [29]. Our results

showed a remarkable difference in plasma FA composition between T2DM and healthy controls. Diabetic patients had significantly higher levels of saturated FAs (SFA), lower levels of MUFA, higher levels of n-6 PUFAs, and lower levels of n-3 PUFA, particularly DHA, with higher n-6/n-3 ratio. That unfavorable profile of plasma FAs was partially explained by nutritional habits. However, the changes we found in 20 and 22-carbon FAs, higher level of AA and lower level of DHA with reduced ratio 20:3/18:2 and 20:4/20:3 indicate possible alterations in activities of desaturase and elongase enzymes. These results suggest an additional unfavorable impact on FA profile in diabetic patients.

Conclusion

Age changes percentage in palmitic acid, palmitoleic, oleic, linoleic acids in plasma phospholipids and MUFA were neither reversed nor prevented by fish oil supplementation while supplementation changes plasma phospholipids profiles in blood plasma elevating ETA, EPA, DPA, n-3 and decreasing percentage of AA. In T2DM patients the percentage of total SFA was significantly higher, total PUFA and n-6 PUFA were significantly higher partic ularly linoleic acid and arachidonic acid whereas n-3 PUFAs, particularly docosahexaenoic acid were significantly lower in T2DM patients. The ratio n-6/n-3 PUFA was significantly higher in observed patients as well as the ratio of total PUFA/SFA.

Acknowledgements

This work was part of the Project of Ministy of Education, Science and Technological Development of the Republic of Serbia III41030

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Vitamin D receptor gene polymorphisms in inflammatory diseases

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Vitamin D receptor (VDR), a member of the nuclear receptor superfamily of transcription factors, is primarily expressed in intestinal epithelial cells and osteoblasts. Its expression in the most of immune cells suggests the possible role of VDR in chronic inflammatory diseases. Single nucleotide polymorphisms (SNPs) located in the VDR gene may influence the synthesis of VDR protein or may be associated with changes in the regulation of transcription process. The role of VDR SNPs in the chronic inflammation is not yet precisely established and available studies of association of VDR SNPs (FokI, BsmI and ApaI) with chronic inflammatory diseases reveal contradictory results. In this study, FokI, BsmI and ApaI SNPs were examined in order to determine their association with chronic inflammatory diseases (bronchial asthma, inflammatory bowel diseases and rheumatoid arthritis). Significant association of FokI with inflammatory diseases was observed, as well as higher F allele frequency in controls compared to patients in all studied groups, indicating lower disease risk in carriers of F allele. No statistically significant differences in the genotype and allele frequency distributionsof BsmI and ApaI were found between patients and healthy controls in all studied groups. However, LD analysis of BsmI and ApaI SNPs showed high level of linkage disequilibrium in bronchial asthma and inflammatory bowel diseases groups. Aditionally, BA haplotype might be considered as the risk factor for inflammatory diseases, especially for bronchial asthma and rheumatoid arthritis, while haplotypes containing "a" allele showed protective effect.

Introduction

Inflammatory diseases include a group of different disorders and conditions that are characterized by inflammation. Unlike an acute inflammation which is protective reaction, chronic inflammation is prolonged, dysregulated and maladaptive response. It involves active inflammation, tissue destruction and attempts at tissue repair [1]. Persistent injury or infection, prolonged exposure to toxic agents or self-perpetuating immune reaction that results in tissue damage, as the main causes of chronic inflammatory processes, are associated with different chronic human conditions and diseases, including atherosclerosis,

arthritis, asthma, inflammatory bowel diseases (ulcerative colitis and Crohn's disease) and autoimmune diseases.

Even the mechanisms of chronic inflammatory diseases' onset are different. In recent years a great attention is paid to vitamin D as a factor that is potentially involved in their pathogenesis. However, the mechanisms by which vitamin D reduces inflammation are not completely understood. Genome- and transcriptome-wide studies showed that vitamin D signalling is involved in different inflammatory responses, including regulation of the expression of genes responsible for the secretion of proinflammatory cytokines, interference with transcription factors involved in the regulation of inflammatory genes or activation of signalling cascades [2]. Moreover, vitamin D enhances the chemotactic and phagocytic response of macrophages, inhibits the surface expression of MHC-II-complex antigen and downregulates the production of proinflammatory cytokines (IL-1, IL-6, IL-8, TNF-α) [3].

Active form of vitamin D ($1,25(OH)D_3$) exerts its biological effects by binding to the vitamin D receptor (VDR), a member of the nuclear receptor superfamily of transcription factors. VDR is primarily expressed in intestinal epithelial cells and osteoblasts, as well as in the other tissues that are not involved in calcium and phosphorus metabolism. Moreover, VDR is located in the most of immune cells such as mast cells, macrophages, dendritic cells, NK cells, T and B lymphocyte [4].

After the binding of $1,25(OH)_2D_3$ to VDR, the receptor undergoes heterodimerization with the retinoic X receptor (RXR) and binds to vitamin D response elements located in the promoter region of vitamin D regulated genes. Conformational changes of VDR-RXR heterodimer induce dissociation of nuclear receptor co-repressor and facilitate the interaction with co-activators [5]. This complex further activates VDR-interacting protein (DRIP) and other co-regulatory proteins that control histone modification, chromatin remodelling, binding of RNA-polymerase II and initiation of the transcription process. Histone acetylation relaxes chromatin structure and transcription of the target genes starts [5].

Vitamin D receptor contains 427 amino acids and 2 main functionally domains: DNA binding domain at the N-terminus and ligand binding domain at the C-terminus [6]. In humans, the VDR is coded by the VDR gene located on 12^{th} chromosome at the position 12q13-14. The VDR gene contains 11 exons, 3 of them (1A, 1B and 1C) are located at the noncoding 5' end, while exons 2-9 code the VDR product [7].

Single nucleotide polymorphisms (SNPs) located in the VDR gene may influence the synthesis of VDR protein or may be associated with changes in the regulation of transcription process. FokI, BsmI, ApaI and TaqI are among the most studied SNPs in the VDR gene. The SNP located in the start codon of exon 2 at the 5' end of the VDR gene is denoted as FokI (f(T)/F(C); rs2228570). It is located in the first of two transcription initiation sites resulting in ATG to ACG change. In the presence of ACG sequence, transcription starts at the second ATG resulting in the synthesis of the protein which is 3 amino acids shorter (424 amino acids) than the protein synthesized in the presence of T allele (427 amino acids) [8]. The shorter protein shows 1.7 fold higher activity compared to

the longer one [8]. Three SNPs that do not lead to any change in either the transcribed mRNA or the translated protein have been identified in the VDR gene: BsmI(G(b)/A(B); rs1544410) and ApaI(a/A; rs7975232) are intronic, located between exons 8 and 9,whileTaqI (t/T; rs731236) is located in exon 9 and leads to a silent codon change (from ATT to ATC) [9]. Compared to FokI which is considered to be an independent marker of VDR gene, BsmI SNP is the mostly studied within the linkage disequilibrium with ApaI and TaqI SNPs. These polymorphisms are linked to further gene variation, which is a variable-length adenosine sequence within the 3′ untranslated region (3′UTR) [9]. The poly(A) sequence varies in length and can be segregated into two groups - long sequences of 18–24 adenosines or short ones. The length of the poly(A) tail can determine mRNA stability, thus the SNPs resulting in long poly(A) tails may increase the local levels of the VDR protein [10].

Previous studies that examined the association of VDR SNPs with chronic inflammatory diseases such as bronchial asthma (BA), inflammatory bowel disease (IBD) and rheumatoid arthritis (RA) showed contradictory results. To the best knowledge of the authors there are no available studies examining the VDR SNPs association with inflammatory diseases in Serbian population. Since the vitamin D signalling may influence different genes involved in inflammation and since the VDR gene variants may influence the vitamin D metabolism, the aim of this study was to examine the association of VDR SNPs (FokI, BsmI and ApaI) with inflammatory diseases (BA, IBC and RA) in the Serbian population.

Patients and methods

A total of 271 subjects were involved in this study. Patients group involved 185 subjects in the mixed inflammatory diseases group consisted of 79 patients with BA (male/female: 32/45; mean age 47.48 ± 15.81), 38 with IBD (male/female: 22/16; mean age 56.33 ± 11.21) and 68 with RA (male/female: 16/52; mean age 58.83 ± 10.34), while control group involved 86 healthy individuals (male/female: 39/47; mean age 44.81 ± 16.78) with no previous history of asthma, atopy or acute and chronic inflammatory or autoimmune diseases. All subjects signed an informed consent. The study was approved by the Ethical Committee of the Faculty of Medicine, University of Niš, Serbia.

SNP detection. Genomic DNA was isolated from the whole blood samples using QIAamp DNA Blood Mini Kit (Quiagen GmbH, Hilden, Germany). The SNPs were determined using polymerase chain reaction – restriction fragment length polymorphism (PCR-RFLP) technique. PCR reaction was performed in the final volume of 25 μl containing 20 ng of DNA, 12.5 μl KAPA2G Fast HotStartReadyMix (Kapa Biosystems Inc, Wilmington, USA) and 20 pmol of each primer. Primer sequences and annealing temperatures are summarized in Table 1. Obtained PCR products were detected by electrophoresis on 2% agarose gel for FokIand ApaIor 1% for BsmI, stained with ethidium bromide and visualized under UV light. Afterwards, restriction digestion at 37°C overnight using FokI, BsmI and ApaI restriction endonucleases (Fermentas GmbH, St.Leon-Rot, Germany) was performed. RFLP products were analysed on 8% (FokI and ApaI) or 6% (BsmI)

polyacrylamide gels stained with ethidium bromide. The interpretation of results was performed according to the size of obtained restriction fragments (Table 1).

Table 1. Primer sequences, annealing temperatures, restriction enzymes and size of fragments generated by FokI, BsmI and ApaI SNPs

SNP (rs number)	Primers	Annealing t [°C]	Restriction endonuclease	Restriction fragments [bp]
FokI rs2228570	F: 5'-AGCTGGCCCTGGCACTG ACTCTGCGTCT-3' R: 5'-ATGGAAACACCTTGCTT CTTCTCCCTC-3'	60	FokI	FF – 265 Ff – 265, 196, 69 ff – 196, 69
BsmI rs1544410	F:5'-GGACCTGTGGCAACCA AGACT-3' R: 5'-CCCGCAAGAAACCTCA AATA-3'	60	BsmI (Mva1269I)	bb – 654, 76 Bb – 730, 654, 76 BB – 730
ApaI rs7975232	F: 5'-CAGAGCATGGACAGGG AGCAA-3' R: 5'-GAGACCTCAGCCATGA GGAGTTGC-3'	64	ApaI	aa– 515, 225 Aa – 740, 515, 225 AA– 740

Statistical analysis. The allele and genotype frequencies, determined in patients and healthy controls, were compared with the values predicted by the Hardy-Weinberg equilibrium using the chi-squared (χ^2) test. Differences in genotype and allele frequencies between patients and controls were tested using $\chi 2$ test or two-tailed Fisher's test when the number of expected cases was small. The differences were considered significant at $p < 0.05$. Genetic risks were assessed by calculating odds ratios (OR) with 95% confidence intervals (95% CI). Linkage disequilibrium (LD) was calculated using Haploview 4.2 software package [11].

Results

The genotype frequency distributions for all studied SNPs are summarized in Table 2. Statistically significant difference was observed in the genotype distribution between healthy controls and BA patients ($p = 0.008$), IBD patients ($p < 0.001$), as well as the mixed inflammatory diseases (BA + IBD + RA) group ($p = 0.001$). No significant differences were found in the genotype frequencies when BsmI and ApaI genotypes of patients with BA, IBD, RA or mixed inflammatory diseases group (BA + IBD + RA) and healthy controls were compared.

Similar results were obtained by the analysis of allele frequencies distribution (Table 3). The allele frequency distributions of the FokI SNP were significantly different between patients with BA ($p = 0.002$), IBD ($p = 0.001$) and RA ($p = 0.041$), as well as in the mixed inflammatory diseases (BA + IBD + RA) group ($p = 0.001$), and healthy controls.

Table 2. Distribution of VDR genotypes in patients with inflammatory diseases (bronchial asthma, inflammatory bowel disease and rheumatoid arthritis) and controls

SNP	Geno-type	C n (%)	BA n (%)	IBD n (%)	RA n (%)	p_1	p_2	p_3	p_4
FokI rs2228570	ff	2 (2.33)	6 (7.59)	1 (2.63)	3 (4.41)				
	Ff	24 (27.91)	36 (45.57)	25 (65.79)	29 (42.65)	**0.008***	**0.0001***	0.099	**0.001***
	FF	60 (69.77)	37 (46.84)	12 (31.58)	36 (52.94)				
BsmI rs1544410	bb	32 (37.21)	28 (35.44)	14 (36.84)	24 (35.29)				
	Bb	36 (41.86)	40 (50.63)	18 (47.37)	27 (39.71)	0.392	0.762	0.836	0.796
	BB	18 (20.93)	11 (13.92)	6 (15.79)	17 (25.0)				
ApaI rs7975232	aa	14 (16.28)	16 (20.25)	9 (23.68)	11 (16.8)				
	Aa	40 (46.51)	41 (51.90)	19 (50.00)	25 (36.76)	0.427	0.413	0.419	0.803
	AA	32 (37.21)	22 (27.85)	10 (26.32)	32 (47.06)				

C – controls; BA – bronchial asthma; IBD – inflammatory bowel disease; RA – rheumatoid arthritis; n – number of cases; p_1 – BA vs. control; p_2 – IBD *vs.* control; p_3 – RA *vs.* control; p_4 – BA + IBD + RA *vs.* control; * – statistically significant *p*-values

Table 3. Distribution of VDR alleles in patients with inflammatory diseases (bronchial asthma, inflammatory bowel disease and rheumatoid arthritis) and controls

SNP	All ele	C n (%)	BA n (%)	IBD n (%)	RA n (%)	p_1 (OR_1; 95%CI_1)	p_2 (OR_2; 95%CI_2)	p_3 (OR_3; 95%CI_3)	p_4 (OR_4; 95%CI_4)
FokI rs22285 70	f	28 (16.28)	48 (30.38)	27 (35.53)	35 (25.74)	0.002* (0.45; 0.26-0.76)	0.001* (0.34; 0.19-0.66)	0.041* (0.56; 0.32-0.98)	0.001* (0.46; 0.29-0.73)
	F	144 (83.72)	110 (69.62)	49 (64.47)	101 (74.26)				
BsmI rs15444 10	b	100 (58.14)	96 (60.76)	46 (60.53)	75 (55.15)	0.628 (1.11; 0.72-1.73)	0.725 (1.10; 0.64-1.92)	0.811 (0.94; 0.59-1.51)	0.911 (0.98; 0.68-1.14)
	B	72 (41.86)	62 (39.24)	30 (39.47)	61 (44.85)				
ApaI rs79752 32	a	68 (39.53)	73 (46.20)	37 (48.68)	47 (34.56)	0.221 (1.31; 0.89-2.03)	0.179 (1.45; 0.84-2.5)	0.370 (1.23; 0.78-1.98)	0.524 (0.89; 0.61-1.28)
	A	104 (60.47)	85 (53.80)	39 (51.32)	89 (65.44)				

C – controls; BA – bronchial asthma; IBD – inflammatory bowel disease; RA – rheumatoid arthritis; n – number of cases; OR – odds ratio; CI – confidence interval; p_1 – BA vs. control; p_2 – IBD *vs.* control; p_3 – RA *vs.* control; p_4 – BA + IBD + RA *vs.* control; * – statistically significant *p*-values

The frequencies of the F allele were significantly higher in healthy subjects compared to patients indicating lower risk for disease (2.2 fold for mixed inflammatory diseases group and BA, 2.99 fold for IBD and 1.8 fold for RA) in carriers of F allele. The distribution of BsmI and ApaI allele frequencies were not significantly different between patients and controls ($p > 0.05$).

Figure 1 represents LD pattern of the analysed populations. A block structure was created for marker pairs with D' > 0.8, using the solid-spine block definition implemented in Haploview. One LD block, containing BsmI and ApaI SNPs, was observed in control, BA and IBD groups. The highest LD was detected in control (D' = 0.91) and BA (D' = 0.91) groups. In the IBD group D' was 0.83, while low LD (D' = 0.76) was observed in mixed inflammatory diseases group (BA + IBD + RA). No LD was observed in RA group. Moreover, FokI SNP was not in LD with other studied VDR SNPs.

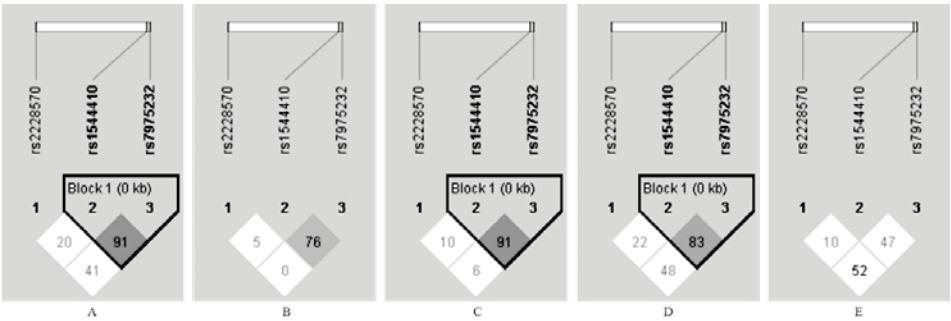

Figure 1. Linkage disequilibrium patterns in the study groups. A – control group; B – mixed inflammatory diseases group (BA + IBD + RA); C – BA; D – IBD; E – RA; rs2228570 – FokI; rs1544410 – BsmI; rs7975232 – ApaI; value in each cell represents D' value; the cells are colour graduated representing the strength of LD between the two markers (darker cells - higher D', lighter cells - lower D').

The distribution of analysed VDR haplotypes is shown in Table 4. Combining BsmI and ApaI SNPs resulted in 4 possible haplotypes (ba, bA, Ba and BA). When each haplotype was tested against all other haplotypes significantly higher risk for the disease was observed in the presence of BA haplotype in bronchial asthma ($p = 0.037$; OR = 1.633; 95%CI = 1.029–2.591) and in the presence of bA ($p = 0.004$; OR = 2.28; 95%CI = 1.283–4.051) and BA ($p = 0.029$; OR = 1.703; 95%CI = 1.055–2.747) haplotypes in RA. Significantly lower risk for the disease was revealed in the presence of Ba haplotype in asthma ($p < 0.001$; OR = 0.074; 95%CI = 0.017–0.317) and in the presence of ba ($p = 0.005$; OR = 0.505; 95%CI = 0.314–0.813) and Ba ($p = 0.012$; OR = 0.361; 95%CI = 0.158–0.824) haplotypes in RA. In the mixed inflammatory diseases (BA + IBD + RA) group, BA haplotype showed increased risk ($p = 0.029$; OR = 1.553; 95%CI = 1.046–2.306), while Ba ($p < 0.001$; OR = 0.178; 95%CI = 0.086–0.369) showed lower risk for the disease.

Table 4. Distribution of BsmI and ApaI haplotypes in patients with inflammatory diseases (bronchial asthma, inflammatory bowel disease and rheumatoid arthritis) and controls

Haplotype	C n (%)	BA n (%)	IBD n (%)	RA n (%)
ba	78 (44.32)	70 (44.87)	35 (46.05)	39 (28.68)
bA	24 (13.64)	25 (16.03)	11 (14.47)	36 (26.47)
Ba	26 (14.77)	1 (0.64)	2 (2.63)	8(5.88)
BA	48 (27.27)	60 (38.46)	28 (36.84)	53 (38.97)

C – controls; BA – bronchial asthma; IBD – inflammatory bowel disease; RA – rheumatoid arthritis; n – number of cases

Discussion

It is well established that vitamin D is involved not only in calcium and phosphorous metabolism, but in many different pathways, including the regulation of genes coding the proinflammatory cytokines and transcription factors involved in the inflammatory response. Single nucleotide polymorphisms located in the VDR gene may contribute to the genetic susceptibility to certain diseases. However, the exact role of the VDR SNPs in the pathogenesis of inflammatory diseases is still under debate.

In this study, the association between three VDR SNPs (FokI, BsmI and ApaI) and inflammatory diseases (BA, IBD and RA) was analysed. Our study revealed significant association of FokI polymorphism with the mixed inflammatory diseases group (BA + IBD + RA), as well as with BA, IBD and RA individually. The frequencies of the FF genotype were significantly higher in healthy subjects compared to patients with mixed inflammatory diseases group (BA + IBD + RA), as well as in BA and IBD individually. Additionally, F allele frequency was significantly higher in controls compared to patients in all studied groups, indicating lower disease risk in carriers of F allele. Tizaoui et al. also showed an association of F allele and FF genotype with the lower risk for BA, especially in patients with low $25(OH)D_3$ levels [12]. In our study, the presence of F allele is associated with the 2.2-fold lower risk for BA. However, in Tunisian and Egyptian population, F allele is associated with the higher risk for BA [13,14]. No association of FokI polymorphism with BA is observed in Chinese Han population [15,16].

Our results showed an association of FokI SNP with mixed IBD group (ulcerative colitis and Crohn's disease), as well as protective effect of F allele indicating 2.99-fold lower risk for IBD in the carriers of this allele. Results of Naderi et al. and Simmons et al. are in accordance with our study, showing significantly higher frequency of ff genotype in IBD patients compared to controls [17,18]. Also, Xue et al. found that ff genotype was associated with a significant risk for ulcerative colitis in Asians [19], while Huges et al. revealed higher risk for ulcerative colitis in the carriers of heterozygous Ff genotype [20]. Furthermore, no

statistical significance compared to controls was found in Irish and Chinese Han populations with IBD [20,21].

The study by Maalej et al. showed significantly higher frequency of FF genotype in patients with RA compared to controls in French population [22]. Similarly, F allele is more frequent in Tunisian RA patients, especially female, compared to controls [23]. The meta-analysis that included only three studies, which examined FokI SNP in RA, also showed an association of this polymorphism with RA [24]. Stratified meta-analysis by the ethnicity revealed a significant association between F allele and RA in Europeans [25]. However, studies by Ates et al. and Masi et al. did not reveal any association of FokI SNP with RA and juvenile idiopathic arthritis [26, 27]. On the contrary, even there were no differences in the genotype distribution between RA patients and controls, our study showed significantly higher prevalence of F allele in healthy subjects compared to the patients with RA, suggesting a protective effect of this allele with 1.8 fold lower risk for the RA development.

Contradictory results obtained in the previous studies could partially be the result of different ethnic origin of the subjects involved in these studies. Furthermore, higher prevalence of homozygous FF genotype in the healthy population could be a result of evolutionary adaptation in the human population. This could also be due to the fact that shorter VDR protein (424 amino acids), synthesised in the presence of FF genotype, have 1.7-fold higher activity compared to the protein synthesised in the presence of ff genotype (427 amino acids) [8]. Moreover, presence of the f allele was shown to be associated with the lower VDR expression after exposure to calcitriol [28].

Compared to FokI, BsmI and ApaI SNPs did not show association with the inflammatory diseases in this study. No statistically significant differences were observed in the genotype and allele distributions between patients and healthy controls in all studied groups. However, LD analysis of BsmI and ApaI SNPs showed high LD in BA and IBD groups.

Distribution of BsmI genotype and allele frequencies in BA patients did not show significant differences compared to the healthy subjects in our study. These results are in accordance with the studies in Chinese population and in Cypriot adolescents [16,29,30]. Maalmi et al. showed significant difference in the genotype distribution in children with BA compared to healthy controls and increased risk for BA in the presence of B allele in Tunisian children [13], while Poon et al. suggested that b allele is associated with the BA in the subjects of French-Canadian origin [31]. Saadi et al. showed a significant association of ApaI with asthma and increased risk for BA in the carriers of minor ApaI allele in Chinese Han population, while other studies, including ours, failed to find the association [16,30,31]. However, our study showed that BsmI and ApaI are in strong LD in asthmatic patients. Haplotype analysis revealed 1.6 fold increased risk for asthma in carriers of BA haplotype and protective effect of Ba haplotype compared to other BsmI and ApaI haplotypes. The studies in Tunisian population showed a protective effect of bAT and bat haplotypes [12,13].

Similarly to results obtained in BA patients, no association of BsmI and ApaI SNPs was found in IBD patients in this study. Genotype and allele frequency distributions did not

show differences in IBD patients compared to controls. Our results are in accordance with those obtained in several other studies [20,32]. In contrast to our results, BB genotype of BsmI was more frequent in Jewish Ashkenazi patients with ulcerative colitis [33]. Also, ulcerative colitis was associated with Bb genotype of BsmI SNP, but not with ApaI SNP, in Chinese Han population [21]. The meta-analysis by Wang et al. revealed no association of mixed IBD with ApaI and BsmI polymorphisms, but after stratification by the IBD subtype, the increased risk for Crohn's disease was detected in carriers of ApaIAA and genotypes containing BsmIB allele [34].

Polymorphisms located in the intronic region of VDR gene, BsmI and ApaI, are intensively studied due to their possible influence on mRNA stability. However, differences in mRNA stability between the alleles were not confirmed in later studies [35]. Two SNPs in the VDR gene, BsmI and ApaI, are among the most studied in RA, especially in association with bone mineral density and in RA with associated osteoporosis. It has been previously shown that RA patients carrying the B allele had lower bone mineral density and increased bone loss [36]. Our study showed no significant differences between the vitamin D receptor genotypes and alleles of BsmI and ApaI SNPs. No influence of VDR BsmI and ApaI polymorphisms on rheumatoid arthritis susceptibility was shown in Spanish, German and Korean patients [37,38]. Moreover, previous studies showed no significant differences between the BsmI VDR genotypes or alleles and the presence or absence of rheumatoid factor [26]. Even no LD was observed for BsmI and ApaI SNPs, our results of haplotype analysis revealed significantly higher risk for the RA development in carriers of haplotypes containing "A" allele (2.28 fold for bA and 1.7 fold for BA). Haplotypes containing "a" allele were associated with the significantly lower risk for RA (13.5 fold for ba and 2 fold for Ba).

Differences in the genotype and allele frequencies obtained in the previous studies could be only partially explained by the different ethnicity of the studied populations. The overall conflicting findings may also be due to the phenotypical diversity of diseases and the different study designs. However, the fact that chronic inflammatory diseases occur as a result of complex interactions of genetic and environmental factors suggests that gen-gen and gen-environment interactions, as well as possible inter-individual differences, should also be taken into consideration when association of SNPs with the certain disease is studied. Nevertheless, further studies are required in order to detect other functionally active variations in VDR gene and to explain the specific mechanisms of the relationships between VDR SNPs and chronic inflammatory diseases.

In conclusion, this is the first study that examined the association of VDR gene variants (FokI, BsmI and ApaI) in Serbian patients with chronic inflammatory diseases (bronchial asthma, inflammatory bowel disease and rheumatoid arthritis). This study showed significant association of FokI SNP with inflammatory diseases and protective effect of F allele. BsmI and ApaI SNPs are in strong linkage disequilibrium in BA and IBD patients, and BA haplotype might be considered as the risk factor for inflammatory diseases, especially for BA and RA. Haplotypes containing "a" allele are associated with the lower risk for BA and RA.

Acknowledgements

This study was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia (grant number III41018).

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Biomedical potential of modified steroids

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Thanks to their capability to pass lipophilic membrane, steroids can enter the cell and, after binding to appropriate steroid receptors, express their specific physiological function. Since even minor changes in steroid structures can cause significant changes in biological activity, medicinal chemists modify steroidal moiety in different ways, trying to reveal pharmacologically active compounds, expressing appropriate potent biological activities, different from original steroids, but with lower side-effects.

Introduction

Diversity of steroidal compounds in living organisms, in humans as well, is obvious, which indicates importance of this class of natural products. Lanosterol, synthesized from isoprenoid monomers is the first animal synthesized steroidal compound in the biosynthetic pathway. Cholesterol is 27C steroid that is created from lanosterol in microsomal membrane, by action of enzymes in 19 reactions. Thus, cholesterol synthesis is a complex and energy expensive process, so it is clearly advantageous to an organism to regulate the biosynthesis of cholesterol to complement dietary intake.

Cholesterol is very important for all animal cells: cholesterol is found in every cell in our body and without it our bodies would not function properly. It is a structural component of biological membranes, along with polar lipids, providing a protective barrier. Cholesterol synthesis is under hormonal control and is also inhibited by elevated concentrations of intracellular cholesterol, which acts through covalent modification and transcriptional regulation mechanisms [1]. One of the most important functions of cholesterol is the production of hormones. Cholesterol is stored in the adrenal glands, ovaries and the testes and is converted to steroid hormones. The steroid hormones (glucocorticoids, mineralocorticoids and sex hormones) are produced from cholesterol by alteration of the side chain and introduction of oxygen atoms into the steroid ring system. These steroid hormones perform appropriate physiological functions. Practically, without steroid hormones we will have malfunctions with weight, sex, digestion, bone health and mental status. Cholesterol is also a precursor for the synthesis of bile acids in the liver, needed for lipid's digestion. They are great emulsifiers, thanks to their amphiphilicity.

According to some studies, testosterone, estradiol, progesterone and dihydrotestosterone (DHT) seem to have significant effect on cholesterol level. Estradiol in particular seems to be responsible for keeping good HDL cholesterol elevated, while other hormones, such as testosterone and DHT may raise total and LDL cholesterol. In physiological conditions the body limits how much hormones the endocrine glands secrete. However, by taking steroids as supplements or medicaments, added steroids affect the regulation of biosynthesis of cholesterol and its products – bile acids and hormones.

Great attention is paid to the hormonal status. This is because steroid hormones regulate many different physiological processes in humans, through all phases of life. Tissue-specific enzymes catalyze steroidogenesis, converting cholesterol to the biologically active corticosteroid, androgen, and estrogen hormones. Enzymes belonging to cytochrome P450 enzymes and hydroxysteroid dehydrogenases catalyze adrenal, ovarian, testicular, placental and other steroidogenic processes [2].

Primary goals in the current strategy for the treatment of steroid hormone-dependent diseases, breast- and prostate-cancer in the first order, is blockade of steroid action and/or the reduction of circulating steroid hormones in cancer tissues. In the treatment of androgen-dependent tumors, antiandrogens, as well as 17α-hydroxylase/C17,20 lyase (CYP17A1) and 5α-reductase inhibitors are the most promising therapeutics [3,4]. Antiandrogens used for medical treatment of androgen dependent prostate cancer are non-steroidal molecules. On the other hand, CYP17A1 and 5α-reductase inhibitors used in today's clinical practice are mostly steroidal compounds. Figure 1 shows therapeutics that are mostly often used for the treatment of prostate cancer [4].

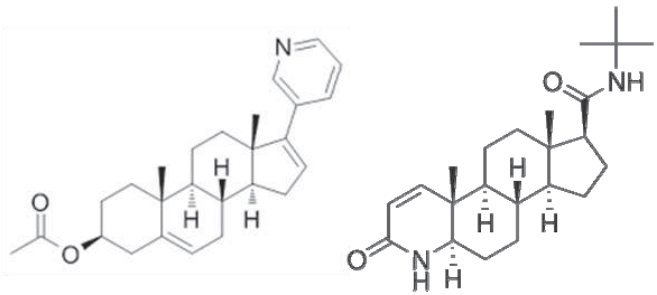

Figure 1. Structures of the most often used steroidal inhibitor of 17α-hydroxylase/C17,20 lyase – Abiraterone (left), and inhibitor of 5α-reductase – Finasterid (right).

Having in mind that estrogen hormones are major threat in the development and growth of breast tumors, estrogen deprivation remains a key therapeutic approach. Endocrine agents are designed to inhibit the production of estrogens or to block its action at the estrogen receptor [3,5]. For many years, tamoxifen, the nonsteroidal antiestrogen that compete with estradiol for estrogen receptor, was the most important therapeutic in the hormonal therapy for all stages of postmenopausal patients with estrogen-receptor–positive cancers.

However, tamoxifen does not completely prevent the action of endogenous estrogen, and this remaining partial estrogen agonist activity is probably responsible for some of its undesirable side effects, such as an increased risk of endometrial cancer [6]. Otherwise, steroidal antiestrogens are in many cases with no estrogenic properties exerted. Such compounds are known as full or pure antiestrogens. Structures of selected steroidal pure antiestrogens are presented on Figure 2 [7,8]. Regardless of the many side-effects, tamoxifen is still the antiestrogen of choice in the most antiestrogen-based therapies.

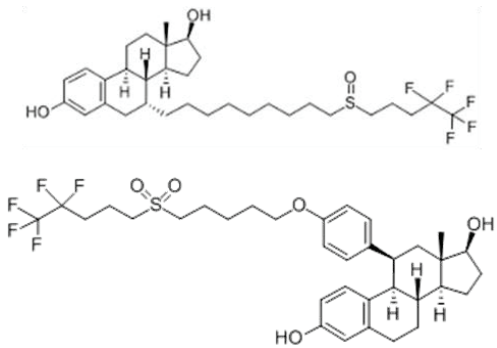

Figure 2. Structures of selected steroidal full antiestrogens: ICI 182,780 (top) and RU 58 668 (bottom).

On the other hand, enzyme aromatase, which catalyzes the key and final step of estrogen synthesis, namely conversion of C19 steroids (androgens) to C18 steroids (estrogens), acts as a master switch in physiological and pathological processes. Apart from the fact that aromatase inhibitors have no partial agonist activity, cancer cells could become resistant to this kind of therapy [9]. Chemical formulas of common aromatase inhibitors are given in Figure 3.

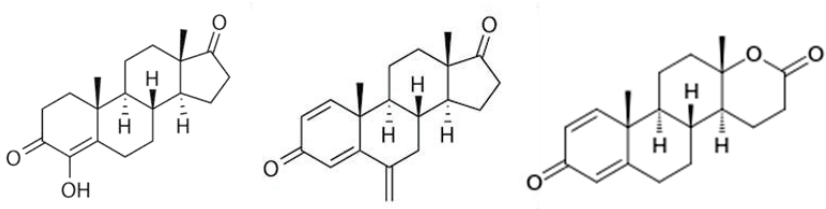

Figure 3. Structures of selected steroidal inhibitors of aromatase: Formestane (left), Exemestane (middle), and Testolactone (right).

Thus, by combining these two breast cancer therapy approaches, the greatest benefit for patients can be realized [3].

Considering the partial agonistic effects of antihormones and the existence of cancer cells resistant to enzyme inhibitors, an important objective of researchers is to find new efficient strategies and/or more effective therapeutics for treating patients suffering from steroid hormone-dependent breast, prostate and cancer of other reproductive tissues, with no side effects or resistance. On the other hand, there are cancers of reproductive tissues that could not effectively be treated with antihormonal therapy due to small or insufficient expression of appropriate steroid hormone receptors. For such malignancies treatment with antiproliferative agents (usually called cytostatics) is recommended.

The modified steroids commonly exhibit biological and physiological properties different from the natural steroids. Therefore, certain compounds of this class are used for the therapy of various steroid hormone-dependent diseases. Based on these data, a broader project, directed towards design and synthesis of potential antihormones, inhibitors of steroid-converting enzymes and antiproliferative agents, resulted in the synthesis of many A, B and/or D-modified steroidal compounds possessing promising pharamacological properties [10-17].

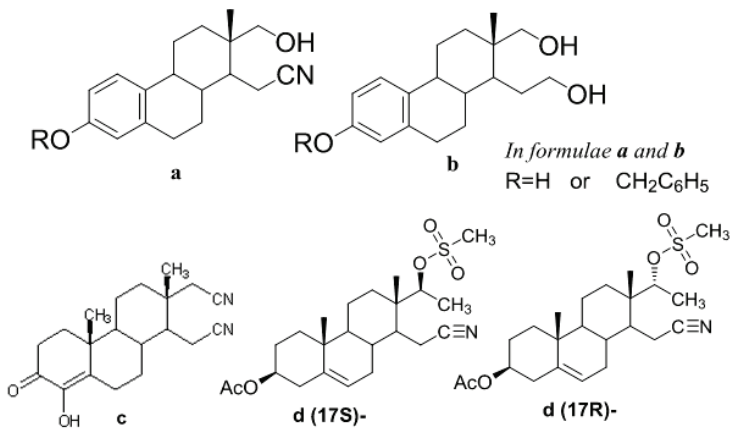

Figure 4. Structures of pharmacologically promising seco-steroids.

Secosteroids

Among other modified steroids, compounds which underwent cleavage of C-C bond in A, B, C or D ring, called secosteroids, are of great importance, because of a variety of their pharmacological potentials. Observed biological activity of many secosteroids isolated from the living organisms, especially marine, emphasize the importance of this class of organic compounds. Secosteroids are important group of modified steroids, which occur widely in nature. Studies on secosteroids showed that modification of the rigid tetracyclic steroidal carbon skeleton by cleavage of the internal C-C bond provides more flexible compounds with new biological properties. The search for steroid compounds analogs with improved biological properties includs ring transformation into seco system. Structures of

seco-steroids that expressed significant pharmacological potential are presented on Figure 4. Among others, 3- hydroxy -17-hydroxy-16,17-secoestra-1,3,5(10)-trien-16-nitrile (a), its 3- benzyloxy or 17-halogen counterpart, as well as 16,17-diole (b) compounds showed practically complete loss of estrogenic activity, while displaying satisfactory antihormonal properties [18]. Further, some 16,17-seco-16,17a-dinitrile compounds approved themselves as strong antiproliferative agents (c), as well as 16,17-seco-17-mesylate derivatives (d) [19], which caused apoptosis in a satisfied amount [20].

Modified steroids with hetero atom in the steroidal moiety

The heterosteroids are of great pharmaceutical importance because of their diverse biological activities. Many of steroidal heterocycles express biological activitis, such as antimicrobial, antiinflammatory, anabolic, antiproliferative activities.

D-homo lactones in androstane and estrane series are steroidal compounds of interest. Androstane derivatives show significant selective cytotoxicity against malignant cell lines, with no effect against healthy cells (MRC-5 cell line). Synthesis, structural analysis and antiproliferative activity of some novel D-homo lactone androstane derivatives [21]. One respresentative of such compounds is presented in Figure 5a. Estrane D-homo lactone (Figure 5b) in molecular docking studies was predicted to bind strongly to estrogen receptor α (ERα), aromatase and lyase, suggesting they could be good starting compounds for antihormonal studies. Its predicted binding is presented on Figure 5c [18].

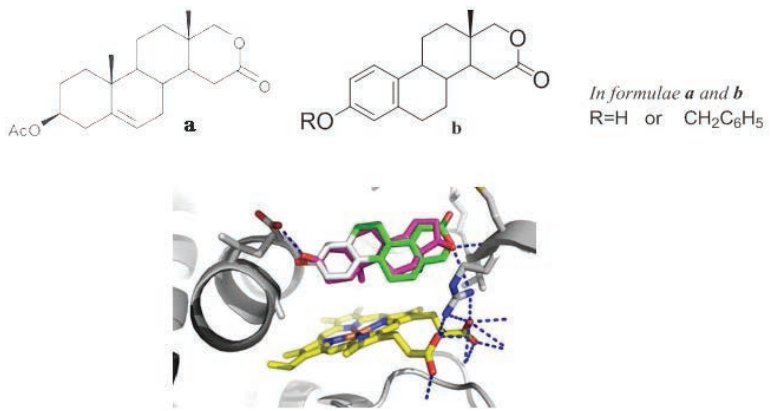

Figure 5. Structures of pharmacologically promising D-homo lactone steroids (a and b) and aromatase docked with 3-hydroxy D-lactone; docking energy: - 10.39 kcal/mol. Note that D-ring lactone forms hydrogen bonds just like androstenedione (magenta).

D-ring coupled heterocyclic steroids belonging to tetrazoles and triazoles (Figure 6) expressed good anttiproliferative effect against estrogen-receptor positive breast cancer cells (line MCF-7), and did not influeced proliferation of normal cells [22,23].

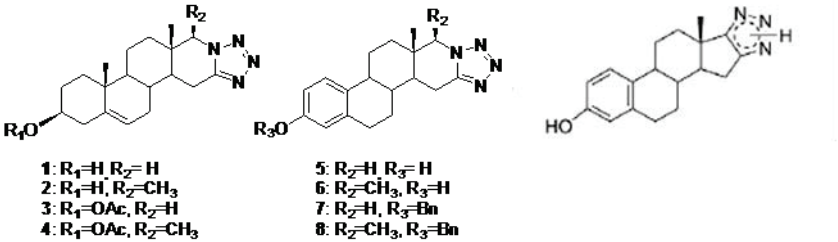

Figure 6. Structures of pharmacologically promising D-homo lactone steroids.

17-Substituted steroidal nitrogen-containing compounds

17α-Picolyl or 17-picolinylidene androstane derivatives express great pharmacological potential. They selectively reduce proliferation of tumor cells, and do not reduce the number of the healthy ones [24]. Some of them induce apoptosis [25], while others inhibit 17α-hydroxylase-C17,20-lyase (P45017α), which is a key regulator enzyme of the steroid hormone biosynthesis in both the adrenals and the testes [26]. Examples are given in Figure 7.

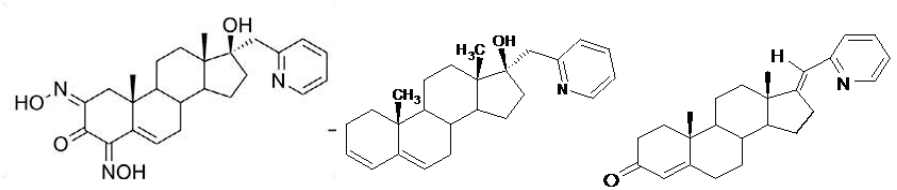

Figure 7. Structures of pharmacologically promising 17-substituted steroidal nitrogen-containing compounds.

In conclusion it should be emphasized that many modified steroids, synthesized by our research group, expressed pharmacological potential in preclinical studies, which is a promising and it is promising to proceed with such research.

Acknowledgements

The authors would like to thank the Ministry of Education, Science and Technological Development of the Republic of Serbia (Grant No. 172021)

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Fructose in the court of justice: good or bad sugar?

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Fructose was present in human diet since the beginning of mankind. Up to the 20th century, humans ingested low doses of fructose which exhibited numerous beneficial effects on human wellbeing. However, modern way of living introduced changes that have greatly affected human health. High-calorie diets and sedentary lifestyle have been correlated to the rising prevalence of obesity, metabolic syndrome, type 2 diabetes and coronary diseases. A shift in diet composition, widespread overconsumption of solid fat and added sugars, especially the use of fructose-sweetened beverages, affected human health in many ways. Considering its specific metabolism, fructose was accused for the rising prevalence of metabolic disorders. However, a more recent data have shown that fructose is not the only suspect, rather one of the accomplices. Moreover, it appears that in the case against fructose some of its favourable properties should be taken into account as mitigating circumstances. If, due to human abuse, fructose readily transgressed from good to bad sugar, the questions that should be answered are: What are the molecular mechanisms underlying harmful effects of fructose overconsumption? At what dose we can put the threshold when physiology shifts to pathophysiology? And finally, is it that bad as it has been anticipated?

Prosecutor's opening statement: Rapid rise in fructose consumption correlates with increasing prevalence of metabolic disorders!

Fructose was always a part of human diet. Free fructose, together with free glucose, is present in fruits and honey, and in smaller amounts in vegetables, while fructose polymers - fructans can be found in some vegetables and wheat. In the past, humans ingested low doses of fructose which exhibited numerous beneficial effects on human wellbeing. The level of fructose consumption remained small until the 19th century, when table sugar became widely available at a low cost due to colonial trade. Nowadays, the typical western diet is rich in sugars and contains on average 50 g fructose/day, thereof approximately 1/6 of ingested fructose comes from natural sources including fruits. The main sources of fructose are sucrose and high fructose corn syrup. Since the introduction of high-fructose corn syrup in 1970s the daily intake of fructose has largely increased. The usage of high-

fructose corn syrup in food industry was encouraged due to its functional advantages over sucrose such as greater sweetness and palatability, better solubility and preservative features, easier handling, as well as low cost and high production efficiency. Due to these favourable properties it became one of the most versatile cost-effective ingredients on the market [1]. To date, high-fructose corn syrup represents approximately 40% of all added sweeteners used in production of soft drinks and fruit juices. Moreover, fructose exhibits numerous useful physical and functional attributes to food and beverage applications, including sweetness, flavour enhancement, colour and flavour development, freezing-point depression, and osmotic stability. Therefore, it is frequently added to foods and drinks in order to enhance palatability and taste. However, increase in fructose consumption coincided with the rising incidence of metabolic disorders [2,3] and fructose was directly accused for the epidemic of obesity. Emerging body of evidence suggests that, due to its specific metabolism fructose affects carbohydrate and lipid metabolism, thus triggering a cascade of events originating in the liver and adipose tissue that can ultimately lead to hyperglycemia, dyslipidemia, hepatic steatosis, abdominal adiposity and insulin resistance, as well as hypertension and hyperuricemia [2,4]. Epidemiological evidence suggest that increased intake of added sugars and/or sugar-sweetened beverages is associated with dislypidemia, insulin resistance, fatty liver, visceral adiposity, type 2 diabetes, cardiovascular diseases, metabolic syndrome, chronic kidney diseases, hyperuricemia, and gout [5-13]. Enhanced hepatic *de novo* lipogenesis, oxidative stress, inflammation and hyperuricemia have been proposed as underlying mechanisms responsible for adverse metabolic effects of fructose [10,14,15]. *Was it always that bad?*

Attorney's opening statement - Fructose is a good sugar! We have made a good deal with nature.

Before 1900. Americans consumed approximately 15 g of fructose per day mainly through consumption of fruits and vegetables. By 1940s, fructose intake had doubled, and rose to 4-fold increase by 1994. Between 2005 and 2010 approximately 13% of adults' total caloric intake came from added sugars [16]. Interestingly, the most recent studies show that estimated dietary sugars intake is either stable or decreasing [17], while the prevalence of metabolic disorders is still rising rapidly. It was suggested that fructose consumption above 20% of energy per day increases risk factors for development of metabolic diseases, while low doses of fructose (less than 36 g per day) benefits glycemic control and might even display beneficial effects on blood pressure.

It was proposed that in the “ancient times” fructose represented an excellent food, due to its capability to stimulate the synthesis of triglycerides and to enhance fat accumulation thus preparing the organism for extended periods of fasting. Moreover, in humans, fructose consumption leads to a rapid depletion of ATP and concomitant rise in serum uric acid due to a loss of uricase. Uric acid (present in form of monosodium urate) is a metabolic product of purine metabolism. In most species, uric acid is metabolized to 5-hydroxyisourate by the enzyme uricase (urat oxidase), and further degraded to alantoin, urea or ammonia, and excreted from the body. However, higher primates, including

humans, have lost uricase. Namely, during Miocene there was a stepwise decrease in uricase activity due to nonsense mutations that have ultimately silenced this enzyme, which led to humans having higher uric acid levels than other mammals. Interestingly, 90% of uric acid filtered by the kidneys is reabsorbed, instead of being excreted, implying that evolution and physiology have not treated it as a harmful waste product, but as beneficial molecule. Importantly, during Miocene, there was an environmental change to a cooler, drier and more seasonal climate. It appears that the loss of uricase provided evolutionary advantage at the time. For instance, due to its effects as an antioxidant and neuroprotector, and also as an inducer of inflammation and activator of the innate immune response, the evolutionary benefit could be the increased life expectancy of hominids. Also, an increase in uric acid level could be one of the mechanisms to maintain blood pressure in times of very low salt ingestion. Interestingly, the increase in uric acid level was even associated with higher intelligence in humans [18]. In light of these, seasonal fructose consumption and concomitant rise in fat stores and uric acid levels might be an evolutionary advantage which has not only enabled human ancestors to survive during times of food shortage, but also helped maintaining blood pressure during periods of dietary change and environmental stress, and participated in intellectual development [19].

Unfortunately, due to marked changes in the dietary habits and marked increase in fructose- and purine-rich foods, these advantages transformed to disadvantages, which appear to contribute to the rising prevalence of various metabolic and cardiovascular disorders [20,21].

Prosecutor's keyword: Breaking a deal with nature… Fructose metabolism

In 2004, Bray et al. [22] argued that increased use of high-fructose corn syrup in the United States mirrored the rapid increase in obesity, and discussed the possibility that the differences in digestion, absorption, and metabolism between fructose and glucose can explain how overconsumption of high-fructose corn syrup in calorically sweetened beverages, and therefore total fructose consumption, may play a role in the epidemic of obesity. The hypothesis was challenged by White and others [23-25], yet, this commentary has raised big attention not only of the scientific community but also of media, general public and policymakers, thus prompting the research toward understanding the metabolic fate of fructose and the mechanisms underlying its possible harmful and/or beneficial effects.

Glucose and fructose are hexoses that differ in the position of carbonyl group. Glucose is a six-carbon aldehyde sugar, whereas fructose represents a six-carbon ketone sugar. This slight structural difference makes a big difference in their biochemical properties. Namely, the digestion, absorption and metabolism of fructose and glucose are different [26]. In the intestine, glucose is absorbed by sodium-glucose cotransporter, while fructose absorption occur further down in the duodenum and jejunum, and is facilitated by a non-sodium-dependent process. After absorption, glucose and fructose enter the portal circulation and either enter the liver, or pass into the general circulation.

Glucose enters cells by insulin dependent Glut-4 transporter. Inside the cell, glucose is phosphorylated by glucokinase to glucose-6-phosphate, from which the intracellular metabolism of glucose begins. Intracellular enzymes control conversion of glucose-6-phosphate to the glycerol backbone of triacylglycerols, among other, through modulation of the rate-limiting enzyme phosphofructokinase. Hepatic glucose metabolism is limited by the capacity of the liver to store glucose as glycogen, regulated by further glucose uptake and by the inhibition of glycolysis including allosteric inhibition of phosphofructokinase by citrate and ATP.

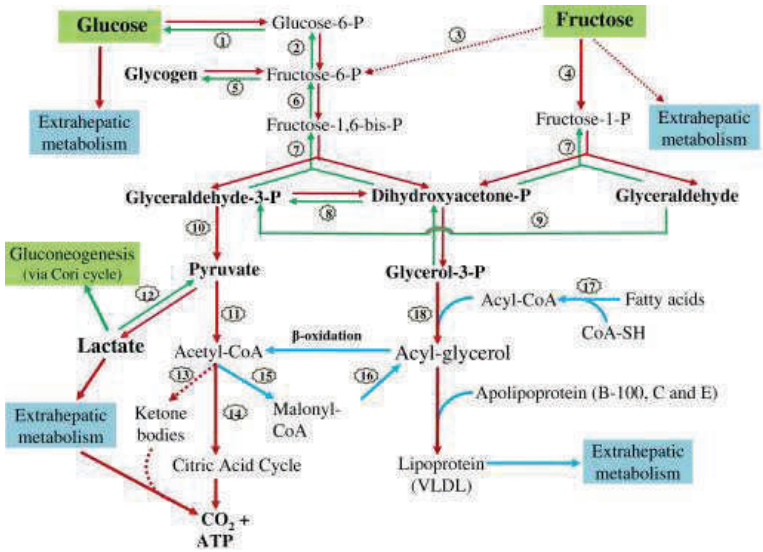

Figure 1. Major metabolic pathways and flux of dietary glucose and fructose. P – phosphate. For enzymes numbered in circles: 1 – hexokinase/glucokinase or glucose-6-phosphatase, 2 – phosphoglucose isomerase, 3 – hexokinase, 4 – fructokinase, 5 – glycogen synthase or phosphorylase, 6 – phosphofructokinase, 7 – aldolase, 8 – triose phosphate isomerase, 9 – triose kinase, 10 – several enzymes including pyruvate kinase, 11 – pyruvate dehydrogenase complex, 12 – lactate dehydrogenase, 13 – ketothiolase and other 3 enzymes, 14 – enzyme group related to citric acid cycle, 15 – acetyl CoA carboxylase, 16 – multienzyme complexes, 17 – acyl CoA synthase, 18 – glycerol-phosphate acyl transferase and triacylglycerol synthase complex. The dashed-line and arrow represents minor pathways or will not occur under a healthy condition or ordinary sugar consumption. The compound names in **bold** represent the major metabolic intermediates or end products of glucose or fructose metabolism (reproduced from [27]).

Liver is the main site of fructose metabolism. The majority of fructose in the portal vein is taken up by the liver to be converted into glucose, glycogen, lactate and in small amount into fatty acids, or to be oxidized within hepatocytes. Fructose enters cells via a Glut-5 transporter irrespective of energy needs or circulating glucose levels. Once inside the cell,

fructose carbons, like glucose, are utilized through glycolysis, gluconeogenesis, glycogenolysis, tricarboxylic acid cycle, lactate production (Cori cycle), pentose phosphate shunt and lipid synthesis pathways, in order to provide substrates for glycogen, amino acids, carbohydrates, fat, ATP, etc (Figure 1). However, fructose and glucose enter metabolic pathways differently. Fructose is phosphorylated by fructokinase to form fructose-1-phosphate, which can directly enter glycolysis. Fructose-1-phosphate can be cleaved by aldolase to form trioses that are the backbone for phospholipid and triacyglycerol synthesis. As the glycolytic pathway becomes saturated with intermediates, these can be converted to glycerol-3-phosphate, providing the glycerol moiety of triglyceride synthesis; they can also be further metabolized to pyruvate, and *via* pyruvate-dehydrogenase to acetyl-CoA and citrate in mitochondria to provide carbon for *de novo* lipogenesis and triglyceride synthesis.

Importantly, in the liver, fructolysis, unlike glycolysis, is neither regulated by insulin nor inhibited by high concentrations of ATP or citrate. Because of its entry point in the liver, fructose bypasses the most important regulatory enzyme of glycolysis, phosphofructokinase-1, which limits further glucose metabolism via feedback inhibition by citrate and ATP. Based on this simplified metabolic pathway analysis, one can assume that fructose serves as an unregulated source of both glycerol-3-phosphate and acetyl-CoA, and enhances hepatic *de novo* lipogenesis. It appears that hepatic production of glucose, lactate, and fatty acids mainly depend on the amount of fructose ingested with a meal or a drink.

However, in everyday life, other lifestyle and dietary factors influence body's utilization of dietary sugars.

Attorney's keyword: Counterclaim evidence - isotope tracer studies

Since fructose metabolism is not dependent on insulin secretion, at least not in the initial steps, and because fructose ingestion causes only a limited rise in glycemia, fructose was initially proposed as a natural substitute of sucrose for diabetic patients. However, the data collected in the past decades, implied that increase in fructose consumption correlates with the rising prevalence of various metabolic disorders, starting with obesity pandemic [2,3]. Nevertheless, the strengths and weaknesses of the supporting evidence are constantly being challenged [23,24,28,29]. Controversies related to fructose-induced metabolic disorders are typically based on the differences between metabolism of fructose and glucose in the liver. Yet, recent isotope tracer studies have provided some interesting evidence regarding utilisation of carbons originating from fructose.

In 2012, Sun and Empie have published meta-analysis of the isotope tracer studies performed on humans and reviewed what tracer studies tell us about the disposal and metabolism of fructose [27]. The results have shown that absorption of fructose alone from the small intestine is slower than that of glucose. Once adsorbed, fructose is primarily delivered to the liver, where it is metabolized for energy and two or three carbon precursors, without dependence on insulin, while glucose is mainly released into the circulation and utilized peripherally. The disposal pathways of fructose and glucose are

different. A significant amount of ingested fructose is oxidised to produce energy (45.8% mean oxidation rate). In resting conditions fructose is utilized in similar amounts as glucose, but under exercise glucose seems to be more preferentially utilized by the body to produce energy. Some of the ingested fructose is converted to glucose within 2-6 hours after ingestion (41% mean conversion rate), and this proportion appears to be lower in obese and diabetic subjects. The data on conversion of fructose to glycogen are limited, but there are studies suggesting that fructose can be a half as efficient as glucose to replenish muscle glycogen after exercise. Although it was reported that a significant amount of fructose could be converted to glycogen in the liver, after conversion to glucose, there are no isotopic traces studies to confirm this statement. Similarly, a significant amount of fructose could be converted to lactate within a few hours (approximately one quarter of ingested fructose), but quantitative metabolic data are still missing. Interestingly, at short time periods (less than 6 hours), only a small percent of fructose carbons are utilized in lipogenesis and converted to plasma triglycerides (less than 1%). While the mechanisms for the fructose-induced hyperlipidemic effect remain controversial, it is possible to assume that in addition to *de novo* lipogenesis, energy source shifting and lipid sparing may play a role [27]. Nevertheless, it appears that the majority of fructose carbons are converted to glucose and lactate, but not to lipids.

Independent expert opinion: Fructose-fed rat – What have we learned from animal model of metabolic syndrome?

Fructose-fed rat represents a commonly used animal model for studying diet-induced metabolic disturbances [30]. Previous studies performed on animals have shown that fructose-rich diet can induce most features of metabolic syndrome, including hypertension, insulin resistance, abdominal obesity, hepatic steatosis, endothelial dysfunction and inflammation [2,31]. Also, it was suggested that oxidative stress participates in the development and progression of these metabolic disturbances [32,33].

Our research group has begun extensive studies on the fructose fed rat, although being aware of the limitations of animal models, such as active uricase in rodents. We have started our research in this field by characterisation of the model. Young 21 day old male and female Wistar rats were subjected to 9-week long fructose rich diet comprising of commercial food and 10% fructose solution instead of drinking water. The dietary regime was applied over the period from weaning to adulthood, since the data on young population, which is at increased risk of developing metabolic disorders in the adulthood, is still missing. The choice of fructose concentration was based on the data that 10% fructose solution closely resembles the intake of sweet solutions characteristic for Western diet [34]. The fructose-rich diet has led to a decrease in chow intake and an increase in liquid and caloric intake. A rise in caloric intake was attributed to passive overconsumption of fructose, since drinking a palatable solution is not causing a feeling of fullness in the way that calories from food do. In addition, increased caloric intake can be related to fructose-induced deregulation of endocrine signals and nutrient detection in the central nervous system, which can delay satiety [35,36]. One can assume that increase in total energy intake

originating from fructose would lead to escalation of body mass, since body mass is crucially dependent on energy balance. Interestingly, in spite of increased caloric intake, total body mass remained unaffected by the applied diet suggesting higher energy expenditure. Nevertheless, fructose-rich diet has increased plasma triglycerides, yet there were no signs of lipid accumulation in the liver. Judging by the literature data, the occurrence and the degree of hepatic lipid accumulation in fructose-fed rats appear to depend to experimental settings (dose and the duration of the dietary regime) as well as susceptibility of rats strains, therefore it should not be considered as usual manifestation of fructose-overconsumption, as compared to rise in plasma triglycerides. Overall, long-term fructose rich diet increases caloric intake without causing changes in the body mass.

Our paper published in 2013 demonstrates that long-term fructose overconsumption induces gender-specific metabolic disturbances in young rats. Male rats appear to be more prone to fructose-induced disturbances in insulin signalling, while female rats develop visceral adiposity [37]. Previous studies on human subjects have also reported gender differences in susceptibility and progression of metabolic disturbances [38,39,40,41]. Although women seem to have higher risk of developing metabolic syndrome and higher prevalence of obesity, less severe metabolic disturbances and/or later onset of adverse phenotypes were observed in females as compared to males. Epidemiological studies indicate that prevalence of insulin resistance-related disorders is higher in men compared to women [39]. Also, men display a higher cardiovascular risk due to differences in prevalence of individual components of metabolic syndrome [38,42]. Studies examining differences in lipid profile patterns between men and women have shown that men tend to have more pathogenic lipid fraction pattern than women, which leads to an increased risk of cardiovascular diseases [38,43].

This initial paper on fructose-fed rats was the beginning of series of investigations aiming to elucidate mechanisms that underlay fructose-induced effects on carbohydrate and lipid metabolism, as well as the reasons for higher susceptibility of male rats to fructose-induced disturbances in insulin signalling that might lead to insulin resistance [37]. Subsequently, we have shown that fructose-rich diet enhances lipolytic processes in visceral adipose tissue of male rats, leading to an increase in plasma NEFA levels [44]. Stimulated lipolysis in the adipose tissue was related to tissue-specific alterations in glucocorticoid prereceptor metabolism, activation of glucocorticoid receptor and consequent upregulation of glucocorticoid-responsive genes involved in lipolysis. On the contrary to males, female rats have developed visceral adiposity without the rise in plasma NEFA level, and the expression of GR was downregulated [45,46].

Liver is the main site of fructose metabolism. Fructose-rich diet was found to stimulate hepatic *de novo* lipogenesis, which was followed by triglyceridemia, but not by lipid accumulation in the liver, both in males and females (unpublished results). In line with high blood NEFA level, an increase in mitochondrial beta-oxidation of fatty acids was observed only in the liver of male rats. Females, in general, due to a larger fat mass as compared to males, might have a more efficient way of removal, processing and storage of excess of lipids, thereby preventing and/or delaying the onset of further diet-induced

metabolic disturbances [47,48]. In line with these, disturbed hepatic insulin sensitivity and low-grade inflammation were present in male [49,50], but not female fructose-fed rats (unpublished results), which might be related to differences in adipose tissue storage function and the plasma NEFA levels. Namely, increased influx of NEFA, derived from the adipose tissue, can promote hepatic insulin resistance and perturb inflammatory signalling [51,52]. Importantly, there were no signs of hepatic steatosis, inflammatory cell infiltration and hepatocytes necrosis in the fructose-fed male rats [50].

Overall, fructose-rich diet induces gender-specific metabolic disturbances in rats, females being more susceptible to visceral obesity and males to disturbed systemic and hepatic insulin signalling and low-grade inflammation. It is possible to assume that enhanced adipose tissue storage function of females might protect the liver from lipotoxicity, thus playing an additional role in later onset of hepatic insulin resistance in fructose-fed female rats. Moreover, the lack of lipolysis in female adipose tissue, shown by unaltered NEFA concentrations, imply its protective role in storage of energy excess. We can assume that young female fructose-fed rats used this protective mechanism against caloric overload also as adaptive measure developed in order to prepare for future pregnancy and successful survival of their progeny.

Oxidative stress was suggested as possible mediator that could induce and/or aggravate diet-provoked metabolic abnormalities including insulin resistance, obesity and fat accumulation in the liver [15,53,54]. Females exhibit better antioxidative capacity as compared to males [55], and this was even correlated with longer life span of females [56]. Gomez-Perez et al. [57] reported that adult female rats on high fat diet, in spite of having a greater excess of body mass than males, showed a less marked insulin resistance profile and better oxidative and inflammatory profile, thus a better capacity to cope with oxidative stress-related disturbances in insulin signalling. In line with these, in our study fructose-rich diet did not induce oxidative stress or affected insulin signalling in the liver of young female rats (unpublished results). Although previous studies have revealed the link between nutritional excess and oxidative stress, suggesting that redox disbalance might participate in the development and progression of metabolic disturbances including insulin resistance-related disorders [32,33,58], the question whether oxidative stress represents a cause of insulin-resistance related metabolic disturbances, remained unanswered.

In attempt to shed more light on molecular mechanisms underlying fructose-related disturbances in insulin signalling and the possible link with redox disbalance, we have focused our later research on male rats. Interestingly, fructose-rich diet has not induced oxidative stress in male rats [59]. Although previous studies provided evidence of direct link between oxidative stress and the insulin resistance, based on our results we can claim that fructose-induced disturbances in hepatic insulin sensitivity occurred in the absence of oxidative stress, and therefore should be attributed to other molecular mechanisms. Yet, the diet has increased the expression of mitochondrial superoxide dismutase (SOD2) [59]. Liver is the main fructose-metabolizing tissue, which rapidly absorbs fructose from the blood and converts it into fructose-1-phosphate due to the high activity of fructokinase. A consequent rapid depletion of ATP and free phosphate in hepatocytes has to be

compensated by the enhanced mitochondrial activity, which was evidenced by increased mitochondrial beta-oxidation of fatty acids in the liver. However, facilitated activity of electron transport chain inevitably leads to promoted electron leakage and superoxide radical anion production [60], therefore the increased expression of SOD2 in the liver of fructose-fed male rats most likely represents an adaptation to site-specific alterations in redox milieu. It is important to point that fructose consumption increased SOD2 expression, but rendered its activity unaffected, and we can only assume that in this situation posttranslational regulation of SOD2 function serves to protect mitochondria from short living superoxide anion radical.

A plethora of data relates mitochondrial dysfunction to insulin resistance [61]. However, there is an ongoing debate whether superoxide anion radical or H_2O_2 is the key player. It is well known that long term exposure of cells to high levels of H_2O_2 leads to insulin resistance [62,63]. However, Hoehn et al. have demonstrated that overexpressed mitochondrial SOD2 has significant insulin sensitizing properties under various cellular and physiological stresses [64]. Since SOD selectively decreases superoxide anion radical levels at the expense of increased H_2O_2 production, the authors pointed to a critical role for superoxide anion radical rather than H_2O_2. Our results cannot offer a direct answer to this question, but we can propose that in our model fine tuning of SOD2 function represents an adaptation aimed to restore hepatic insulin signalling. Namely, although hepatic insulin signalling was deregulated after fructose-rich diet, a clear state of insulin resistance was not induced. Since low doses of hydrogen peroxide can enhance insulin sensitivity *in vitro* and *in vivo* [63,65], while long term exposure of cells to high levels of H_2O_2 leads to insulin resistance [62,63], we can assume that under the conditions of increased production of superoxide anion radical, highly expressed SOD2 enables fast switches in the enzyme activity, where pulsatile rapid dismutation of its membrane impermeable substrate to permeable H_2O_2 could provide positive effect on insulin action aimed to restore hepatic insulin sensitivity.

However the question whether consumption of higher doses of fructose might induce oxidative stress in the liver and consequently contribute to development and aggravation of metabolic disturbances in later adulthood, remained unanswered. In light of previously reported sex differences in antioxidative capacity, it is possible to assume that high doses of fructose might lead to more pronounced redox-related disturbances particularly in males. To answer this question, we have subjected male rats to long term high-fructose diet regime.

We hypothesized that 60% fructose solution would induce more prominent effects on metabolism of young male rats, since males are shown to be more prone to development of diet-induced metabolic disturbances such as insulin resistance-related disorders. Interestingly, the effects of high fructose diet were not as intense as we have anticipated. Namely, there were no signs of hepatic lipid accumulation and steatosis or clear state of insulin resistance [66]. High-fructose diet has led to an increase in caloric intake but the body weight and liver mass remained unaffected, which is in accordance with results obtained from another animal study using 60% fructose enriched diet [67]. Indeed, this diet increased

hepatic *de novo* lipogenesis, induced hypertriglyceridemia and visceral adiposity which were not followed by increased plasma NEFA level. This resembles the situation observed in female rats subjected to moderate fructose-rich diet. Still, while female rats preserved systemic and hepatic insulin signalling, without low-grade inflammation, a slight disturbance in hepatic, but not systemic insulin sensitivity was recorded in male rats subjected to high fructose diet [66]. Since plasma NEFA levels in high-fructose fed male rats remained unaltered, as well as hepatic mitochondrial beta-oxidation of fatty acids, the decrease in hepatic insulin signalling might be attributed to low-grade inflammation, but not to oxidative stress [65,68]. Namely, markers of general redox conditions and lipid peroxidation remained unaltered. Again, the dietary regime has affected SOD2 function, but this time SOD2 activity was increased while the expression remained unaltered [68]. The enhanced SOD2 activity most likely represents transient modulation of the enzyme function in response to site-specific alterations in redox conditions which might reflect current energy demands of the cell.

Overall, fructose-rich diet induces gender-specific metabolic disturbances in young rats. Males were shown to be more prone to insulin resistance-related disorders; however the disturbed hepatic insulin signalling could not be attributed to oxidative stress, at least not at the young age. The lack of oxidative stress observed after both moderate and high-fructose diets imply that young males are capable to maintain the redox homeostasis challenged by the energy overload and protect the cell from oxidative damages. However, the possibility that prolonged fructose overconsumption might finally lead to oxidative stress and consequently contribute to progression and aggravation of metabolic disturbances in later adulthood, should be further investigated.

The questions arise: Why neither moderate nor high fructose-rich diets have induced oxidative stress in the rat liver?

Not so bad, as we expected! Prooxidant/antioxidant properties – favourable amendment…

The proposed causative role of oxidative stress in induction of fructose-induced metabolic disturbances was not confirmed in our studies, since both moderate and high-fructose diet decreased hepatic insulin sensitivity and induced low grade inflammation in male rats without the occurrence of oxidative stress. One of the possible explanations might be related to fructose-specific antioxidative/prooxidative properties. Namely, the relation between fructose consumption and oxidative stress appear to be rather complex, since fructose was shown to produce both pro- and anti-oxidative effects, depending on the dose, duration of consumption and (patho)physiological milieu [69]. Current literature data suggest that fructose exhibits antioxidative and protective effects after short-term application, while its long-term consumption exerts mostly negative effects; however our results on young fructose-fed rats do not support this view. The absence of oxidative stress observed after long-term fructose-rich might be, at least in part, attributed to young age of the animals. However our preliminary results on adult fructose-fed rats do not support this assumption. A more plausible explanation could be related to the unique metabolism of

fructose. Namely, fructose and its metabolic derivatives such as fructose-monophosphates and fructose-bisphosphate show high antioxidative capacities, therefore rapid and unregulated fructose phosphorylation can *in situ* provide high amounts of potent cellular antioxidants to scavenge free radicals. Importantly, fructose and its phosphates in particular appear to be better antioxidants as compared to other sugars such as mannose and glucose [70,71], consequently the effects of different isocaloric diets might differ. Apparently the hypothesis that adverse diet-induced metabolic disturbances should be attributed to excess of energy intake and to changes in body weight should be reexamined. In addition to high antioxidative capacity and cytoprotective effects, fructose promotes the production of reducing agent NADPH by pentose phosphate pathway. It appears that fructose stimulates anabolic pathways such as lipogenesis in the liver, but the absence of more aggravated metabolic disturbances in high fructose fed rats might arise from the absence of marked oxidative stress due to antioxidative properties of fructose and its metabolic derivatives.

Yet, the situation in human population seems to be more complex since humans lack active uricase and high uric levels affect metabolism in many ways including disruption of mitochondrial function. One has to carefully interpret data obtained in animal models, especially in attempts of transferring this knowledge on human population.

Bad sugar or good sugar? Simple question, simple sugar, complex answer….

Since the ancient times, fructose has exhibited numerous favourable properties, and consumption of low doses of fructose appears to be beneficial for human health. However, studies on human subjects support the relationship between fructose overconsumption and rising prevalence of metabolic disorders. Nevertheless, the question why the prevalence of metabolic disorders is still rising despite decreasing sugar intake remains opened. Apparently, we cannot blame it all on fructose. Moreover, the situation is not that bad as we have expected, since the absence of more aggravated metabolic profile could be attributed to antioxidative properties of fructose and its metabolic derivatives. If one decides to accuse fructose for epidemic of metabolic disorders than the verdict should be murder by negligence rather than premeditated one.

Acknowledgements

This work was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia, Grant III41009.

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Vanadium speciation detection by synchrotron based X-ray absorption spectroscopy: application of XANES in biological systems

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Challenges in investigations of V in biological materials date from the end of the 19th century. Difficulties in the speciation of this transition metal originated from its complex physical-chemical properties, and are further complicated by complexity of cellular structures. Traditional spectroscopic techniques employed in describing V behavior, are mainly limited to array particular oxidation state of the element. XAFS represents the unique method for determination of structural properties of V in biological samples, which at the same time elucidates properties of all V species present. Dependent of energy, two XAFS regions are distinguished. Majority of information, necessary for comprehension of V metabolic and detrimental effects in bio-systems, are obtainable by X-ray absorption near edge structure (XANES) spectroscopy by which the valence and local atomic geometry could be revealed. This technique is referred to the spectral range from -30 eV to +50 eV with regard to absorption edge energy. At higher energies, up to 1000 eV from the bound energy of V core level electron, where the effects of multiple elastic scattering from other atoms dominate, distance and identification of the nearest metal neighbors could be determined by EXAFS (Extended X-ray absorption fine structure). The special advantage of these two methods results from the production of synchrotron based X-rays and the possibility of measurements in disordered and diluted samples, typical for biological fluids, cells and tissues. Development of numbers of programs effective in analyzing of XAFS spectra, and the enhancement of quality of synchrotron-produced X-ray, leads to significant progress in investigations of V in living systems. XANES, as the more informative XAFS-technique, becomes a method of choice for structure investigation of many transition metals, including V, in biological media. Published results were dealing mainly with unraveling the features of pre-edge spectral region which consist the majority of useful information for V speciation. Since the pre-edge peak could originate from the combination of various V oxidation and symmetry forms, their contributions were determined by employment of various modern software programs. In this sense, determination of the intensity and the position of pre-edge peak constituents are of crucial importance.

Introduction

Vanadium is widely spread, naturally occurring, multivalent transition metal, essential in micromolar concentrations for many eukaryotic organisms, but at higher concentrations it is toxic and can represent an environmental threat. This element has a range of oxidation state from -1 to +5, and different coordination geometry with various ligands [1]. Among them, of particular importance are compounds in oxidation states +4 and +5 that are the most stable in physiological conditions and concentrations between 0.1–1 mM. V^{4+} might oligomerize under physiological conditions at concentrations above 10^{-6} M, oxidize in the presence of oxygen (under aerobic conditions), or generate complexes in the presence of phosphates, sulfates, thiols, or carboxylic acids [2]. V^{5+} might also oligomerize (dependently of concentrations and pH), create complexes or being reduced in the presence of reducing agents such as gluthatione, ascorbate or NADPH [2]. In these reactions, besides changing the oxidation state, V often changes coordination environment. Knowing that different V species exhibit various biological effects, above mentioned reactions could have considerable influence on V activity. Therefore, the attention is focused on the reactions of biologically active V^{4+} and V^{5+} complexes with organic ligands present in live systems [3-5].

Advantage of XAFS (imperfection of spectroscopic methods)

The common spectroscopic techniques used for elucidation of vanadium properties in biological matrices are directed mainly to determination of concentration, oligomeric forms and the effects of the one of the physiologically important vanadium species [4]. In order to yield a good experimental set of data, a milimolar concentration of sample which contains V is required. In addition, methods based on the separation of V-bound biomolecules by chromatographic or electrophoretic techniques often lead to unreliable results, due to the generally low stability of V-biomolecule adducts under the conditions in such analysis [6]. X-ray absorption fine spectroscopy (XAFS), on the other hand, allows simultaneous detection of all, rather than only one, oxidation form of V and their detailed speciation [6]. Because of its element specificity and no requirements for any long-range order in a sample, XAFS enables recording of elements in the native physiological environment [7-9]. But first of all, since XAFS is very sensitive technique, a small (micromolar) concentration of desired element could be successfully examined.

About XAFS

The discovery of XAFS as a technique able for deep characterization of physical-chemical properties of elements relevant in various branches of natural sciences was related with availability of intense X-ray synchrotron sources in the mid-1970s [10]. From than XAFS has become one of the most important methods for elucidation of the local structure of particular atoms in a wide variety of disciplines [4]. To reach detection limits and adequate energy resolutions for spectra of biologically relevant elements, this technique requires the availability of synchrotron radiation sources [11].

Synchrotrons are huge and complicated (ellipsoidal shape) facilities which are able to accelerate electrons up to the relativistic speeds, and generate X-rays with specific spectral characteristics (Figure 1). Constructed to produce the rays in the broad energy range, with a high brilliance (number of photons emitted in the narrow spectral range per unit time and solid angle), small vertical angular divergence, high degree of polarization, pulsed time structure and high brightness (photon flux per solid angle), it has much more possibilities to satisfy the XAFS experimental conditions than the laboratory X-ray tube aperture. In synchrotron, the kinetic energy of electrons, produced in other type of accelerators such as LINAC, abruptly increase by passing the group of electrons (bunches) through booster ring. Booster synchrotron accelerates the electrons up to desired energy (several MeV) after which that they are transferred to the storage ring.

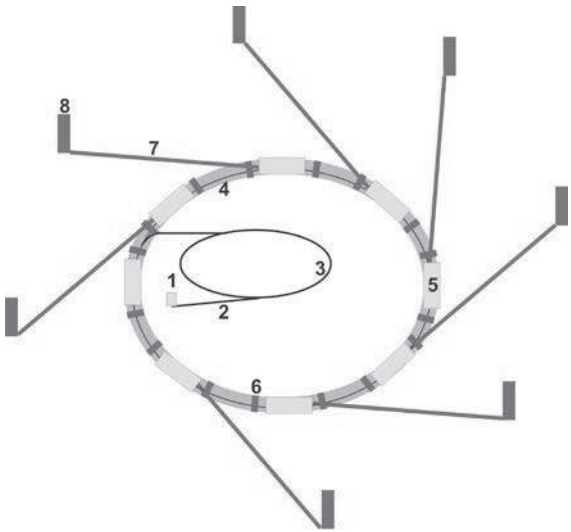

Figure 1. Shematic view of synchrotron with the crucial structural elements: Electron gun (1), Linear accelerator (2), Booster ring (3), Storage ring (4), Bending magnets (5), RF cavity (6), Beamline (7) and End station (8).

Storage ring is the circular evacuated pipe in which the electrons, under the ultrahigh vacuum, are forced to follow circular path under the action of magnetic fields of bend magnets which are distributed along the circumference. Electrons are accelerated whenever their path bends, emitting the radiation. The emitted X-rays than enter the beamline, the long pipe-instruments that safely deliver X-rays to the experimental chamber. Beamlines prepare the beam for the particular experiments by precollimating, splitting, filtering, monochromating and focusing the beam by a wealth of the modern optic instruments. The most of the XAFS experiments require an intense beam of order 10^{10} photon/s within required energy bandwidth of en eV to obtain good data. In addition they need also the rays with tunable energy obtainable by far only in synchrotron. XAFS could be measured in transmission or fluorescence geometries. For concentrated samples (higher than 10%,

without a pinholes with a grain of powder no bigger than the absorption wavelength) XAFS is recorded in transmission mode but in the case of tick samples or small concentrations, the transmission mode is no more as effective due to the low signal-to-noise ratio. Therefore, fluorescence detection is used for analyzing of V in biological materials and diluted solutions. Measurements in fluorescence mode are typified by the appearance of fluorescence lines of other elements in the sample as well as the lines originated from elastic and inelastic scattering. For the assay of V in biological samples fluorescence detection is preferable because its concentration could not exceed mM/L. However, obtained results are compared with a XAFS of standard (commercial) V compounds commonly recorded in transmission mode. As a result of high intensity of X-ray beam, photoreduction of V represents the serious problem which could be overcame by shortening dwell time (the time spent with the beam focused on one point [12], increase of dwell time lead to the increase of X-ray flux-number of photon which reach the sample and which cause sample damage) or by protecting of samples. Hence, samples should be/are often cooled with a liquid nitrogen cryo-jet.

Physics of XAFS/Short theory

The basic physical quantity measured by XAFS is the X-ray linear absorption coefficient (μ), which shows how deep the X-ray photon enters the atom[11]. XAFS describes modulation of the linear absorption coefficient near and above x-ray absorption edge. Absorption edge is referred to the promotion of the core-level electron to the continuum.

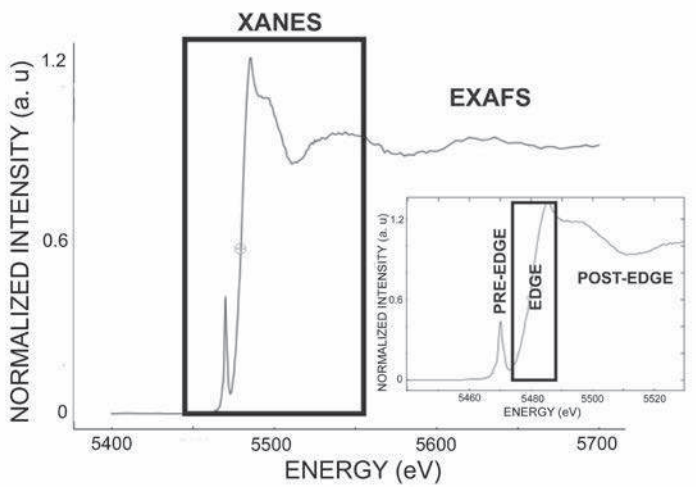

Figure 2. XAFS spectrum of V in *P. blakesleanuus* with designated XANES and EXAFS region. Inset: the XANES part of the spectrum, divided to three distinct regions.

The typical XAFS spectrum, shown in Figure 2, could be divided into two regimes: X-ray Absorption Near Edge Structure (XANES) which comprises approximately the region of

50 eV below and above absorption edge, and Extended X-ray Absorption Fine Structure spectroscopy (EXAFS) which deals with the behavior of X-ray coefficient at the higher energies (up to 1 keV above the absorption edge). Physical basis of both spectroscopic technique are the same, but the information they provide are different. Namely, XANES give us the information about oxidation states and coordination geometry while the identification of the nearest atoms and their distance from the central atom are the subject of EXAFS measurements. In both type of experiments, after interaction of atom with the high-energy photon, the ejected photoelectrons have leaved it reaching the neighbors atoms. Photoelectrons waves scatter from the closer atoms creating the interference between the outgoing and scattered wave. This leads to energy-dependent oscillations (fluctuations) in the X-ray absorption probability reflected through variations in the value of μ. These oscillations are consisted of the narrow and intense peaks in the immediate vicinity of absorption edge and together with the examination of the behavior of μ below the absorption edge represent the subject of XANES spectroscopy. At higher energy, oscillations are less pronounce, peaks are broader and less intense (EXAFS region (Figure 2)). Dependently of the type of information which are required for resolving some problem in the particular case, it is very important to pick out the XAFS technique. Since the most of physiologic effects of V stem from its oxidation states and local symmetry, XANES is the more informative method and therefore is the subject of this review.

Sample preparation

Maintaining of crucial (vital) cell characteristic prior to, and during the experiments is very important. The preparation of samples is a relatively simple and the most important step is to prevent degradation of tissue when the cell is relocated from its natural environment, and to arrest physiological processes that occur in examined organism. Therefore, sample processing has to be followed by immediate fixation. In this treatment, the homogeneity of samples has to be conserved. Biological samples are often liquid and satisfy the requirement of homogeneity. However, inhomogenities of samples could appear during the experiment as a result of phase separation and radiation damage. Namely, intense beam could induce the photolysis of water which further produces the holes in the sample. Concerning indicated problems, the most efficient way for obtaining a suitable sample for experiment is cryofixation *i.e.*, freeze-drying of hydrated specimen (lyophilization). Cryofication is the process which is also suitable to preserve examined material for convenient transport and must be carried out immediately after sampling. To minimize ice formation, sample should be blotted with filter paper before rapidly shock freezing in liquid ethane, and chilled with liquid nitrogen to 77 K.

Basis of XANES theory

The high sensitivity of XANES spectroscopy to the oxidation state and coordination geometry of metal ions [1], makes this technique particularly attractive for determination of V speciation, as it occurs in nature in a redox equilibrium of three oxidation states (V^{5+},

V^{4+} and V^{3+}), and in a variety of coordination environments [3]. These make this technique more important than EXAFS because all V metabolic actions are exclusively linked to its oxidation state and coordination geometry. As it was already mentioned, XANES refers to the region of ± 50 eV from the absorption edge, and represents/appears as a sharp increase of the absorption coefficient due to ejection of a core level electron to unoccupied bound states or to the continuum. The main edge position is shifted towards higher energies with increasing valence, but as the energy difference between absorption edges of two different vanadium oxidation states is rather small, and multiple scattering features dominate the structure of the edge, this approach of valence determination can be misleading [13]. On the other hand, high-resolution vanadium K-edge absorption spectrum reveals characteristic shape, positions and intensities of pre-edge peaks, which depends on the valence and symmetry of examined V species [14]. These pre-edge features are the result of dipole 1s-3d transitions with small contributions of the weak 1s-3d quadrupole electron transitions[15]. Such spectral features based on bound-to-bound transitions are common for all transition elements, because of strong hybridization of their 3d-4p orbitals, as well as the mixing of 3d orbitals of the metal and 2p states of the ligand [1, 15]. For vanadium complexes with a regular octahedral symmetry, the pre-edge features are rather weak, since the 1s-3d transition is strictly forbidden for species with a center of inversion symmetry [1]. Distortion of symmetry and increase of valence makes the 1s–3d dipole transitions allowed, due to low-lying empty states that derive from directly or oxygen-mediated 4p-3d intersite mixing in vanadium oxides [1,16]. This causes an increase of the peak intensity [15]. The ability of vanadium to undergo redox and coordination changes in various (often similar) conditions [16], requires identification of its symmetry and valence for every particular case. XANES is the only method enabling parallel determination of multiple vanadium species present in an investigated system [17].

Until now, XANES spectroscopy was used mostly for the determination of vanadium speciation in V-doped catalysts [18], nanomaterials [19], industrial by-products [14], mineral samples [13], V containing enzymes [20], as well as biotransformation and biodistribution of vanadium-containing anti-diabetic drugs [21]. Investigations of vanadium valence and symmetry in living organisms are very scarce and limited mostly to V-accumulating sea organisms (e.g., tunicates, *Pseudopotamilla occelata*) [22]. The coordination chemistry of vanadium in the bromoperoxidase enzyme of the algae *Ascophyllum nodosum* has been studied in its native forms[20]. In these studies, the clear distinction between various vanadium species and forms were observed. However, in the recent time determination of physical and chemical characteristic of various atoms and molecules in biological samples *in vivo* (*in situ*) represents the particular challenge for many scientists. That is because in living cell, except of many V species (oxidation states) possibly present in sample, its distribution is often /could be different in to one or more specific cellular compartment. Further, every compartment could represent system *per se* which manifests various response toward the same element. Therefore, the micro-X-ray fluorescence imaging (XRF) of whole sample (achieved by irradiation of the sample with the X-rays close to absorption edge of atom) is necessary to identify the place(s) of accumulation of examined

atom. This is the roughly scheme of the distribution of particular atom in cell/sample without going deeply into the matter of its structural characteristic (valence, symmetry, type of ligand and its distance). Recently, several studies have demonstrated the use of X-rays for trace element mapping in different cell types [5,23,24] (Figure 3).

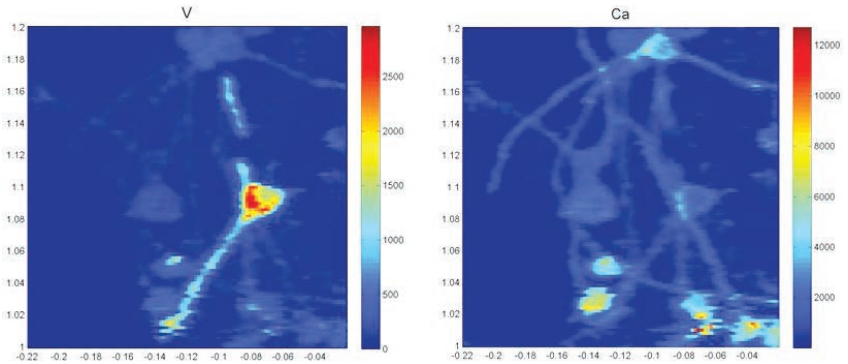

Figure 3. X-ray fluorescence maps of V and Ca in the mycelium of *P.blakesleeanus* treated with V^{5+}.

XANES spectra

The spatial distribution of the atom of interest, determination of regions with various concentrations can be mapped in XANES experiments from every pixel.

XANES spectra are usually plotted in the form of normalized intensity of μ versus energy. Normalization of the intensities of linear coefficient is a procedure for adjustment of obtained data with regard to the various fluctuations that appeared in a sample due to thickness, sample preparation, detector functioning etc. It is very important step enabling us to qualitative compare the absorption properties of various V compounds. Every XANES spectrum could be divided in the three parts: pre-edge, edge and post edge region (Figure 2 inset). As it is mentioned in the previous paragraph, pre-edge peak is the most informative region for transition elements. This is followed by an abrupt increase of μ with a weak shoulder (absorption edge) on the absorption curve culminating, in the case of V, at about 5484-5 eV. The energy position and the intensity of pre-edge peak are determined by oxidation state and local geometry around absorbing V atom. The peak position is shifted toward higher energy if the oxidation state of element increases. That means that the center of the pre-edge peak of V^{4+} compounds is located at lower energy than that of V^{5+} but at the higher with regard to the position of V^{3+} pre-edge peak. Distortion of symmetry is followed by the increase in intensity of pre-edge peak of V. Therefore, the atoms with octahedral coordination geometry (with inversion center of symmetry) produced a pre-edge peak of lower intensity than the atom with distorted square geometry. The highest pre-edge peak is a result of the existence of tetrahedral-coordinated V species. XANES data of main vanadium complex are accessible from literature, and serves as a “control of

the control" [1,13,17]. Furthermore, recently published library of XANES spectra of some biologically most relevant V-complexes considerably relieve identification of unknown V-compounds in biological fluids, cells and tissues [17]. The most present V compounds are vanadate and vanadyl (as a sodium ortho vanadate and vanadyl sulphate). The spectrum of sodium orthovanadate is characterized by the highest pre-edge peak (together with also tetrahedral vanaditine (V^{5+})) comparing to all other V^{5+}-containing compounds. This originates from its tetrahedral geometry (Figure 4, the most upper spectrum, V^{5+} st).

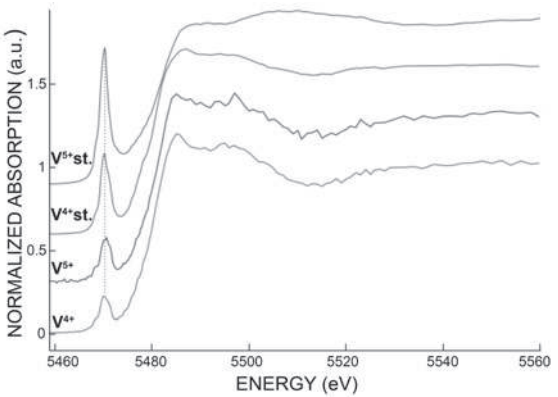

Figure 4. XANES spectra of two common V standards and V compounds produced in biological organism after treatment of the standards.

The intensity and position of V^{4+} (vanadyl sulphate) pre-edge peak depends on the number of surrounding water molecules. In the presented example, the spectrum of vanadyl sulfate of distorted octahedral symmetry was measured (Figure 4, the second spectrum from the top, V^{4+} st). Although XANES is the most reliable techniques in V speciation, there are only several studies using this technique in describing 'state' of V in bio-systems. Thus, the accumulation of V^{3+} in whole blood cell from *Ascidia ceratodes*[22] was first obtained by XANES. By the same technique, several years later, presence of V^{3+} surrounded by six oxygen atoms was documented in the living ascidians blood cells [25] and *P. occelata* [26]. This V oxidation form is characterized by very low, almost negligible pre-edge peak (indicating almost ideal symmetry with an inversion center) located at the energy of 5468.4 eV. The first structural examinations of bromoperoxidase enzyme from *Ascophylum nodosum* elucidated only the substantial alteration in the oxidation state and symmetry between reduced and native enzyme form [20] but their exactly characteristic by considering of pre-edge peak were not puzzled out at this moment. Collected spectra were featured by the existence of intense pre-edge peak, characteristic for the noncentric V-O (double) bonds. In the later XANES study of the same enzyme the distorted octahedral structure of V^{5+} in the enzyme was established [27]. Similar pre-edge feature was absent in the XANES spectra of the mushroom from the genus *Amanita*, well known to accumulated of non-oxo form of V^{4+}-amavadine [20].

In contrast to the mentioned XANES biological investigations where V state is pretty clear, there are investigations dealing with vanadium in several oxidation and/or symmetry form (Figure 4, the spectra marked as V^{5+} and V^{4+}). In such cases, previously experience in the determination of the contribution/share of various vanadium species, concerning both oxidation state and local geometry, had to be taken into account [9,13,28]. The great progress has been made in the investigations of the models of presumed V complex in biological systems [17]. Our recent investigations of vanadium coordination structure in the mycelium of the fungus of *P. blakesleaanus* contained detailed XANES analysis of complex (but reach and informative) spectra, that required additional statistical and analytical processing (interpretations) [5].

Preedge peak analysis

The analysis of the pre-edge spectral characteristic has to be carried out to resolve the structures of accumulated V, particularly when it is present in more oxidation and symmetry forms. In live systems coordination environment of V could deviate from the geometry of commercially known V compounds. In this sense, normalization of the spectra has to be carried out [29]. Detailed description of the normalization process is presented in the paper of Wong et al. [1]. Percentage of V-mixed oxidation state and/or symmetry dominantly contributes to the spectral features of XANES spectra. The main parameter which is necessary to be determined are the "pre-edge peak intensity" (maximum intensity of the net pre-edge peak), "total pre-edge peak area" (sum of the integrated area of each component) and the "pre-edge peak centroid energy" (area-weighted average of the position in energy of each component) [13]. Very often the energy position of the point of interest with highest intensity corresponds to the centroid energy, but there are the cases where it is not a true because of the very close energies of various V species, and if so, the analysis should be done by the comparing of integrated-area values [13]. The first data about mixed V species obtained by XANES were obtained in mixture of two pure V compounds in various percentages [9,13,28]. In biological samples this could be even more complicated as more cellular compartments could accumulate V in more V oxidation and symmetry forms[5]. Therefore, the appropriate spectral analysis should to be conducted. Very important step is the deconvolution using different programs such as ATHENA [29]. Discrete transition, such as promotion of electron to higher energy level should be fit with Lorentzian function corrected by the factor comprising the resolution function of monochromator. For this reason, the pre-edge features are modeled/fitted by pseudo-Voigt function, because of the both Lorenzian and Gaussian contribution [13]. The edge step is approximated by arctangent function (Figure 5).

Performing this mathematic process, it is worth to know the spectral characteristic of the basic V species. For example, although vanadate and vanaditine has the same oxidation state and coordination geometries, their pre-edge peaks are similar but not the same. Given that the accumulation of V in live systems could be rather complex due to an existence of various species in different cellular compartments, appropriate choice of possibilities, offered by the software, has to be selected to give us the best fit of recorded spectrum. The

several values of pre-edge peak centroid energy and intensities for some V compounds which concern the biological systems as well as the data of V with various structural features are available from the literature [5,17].

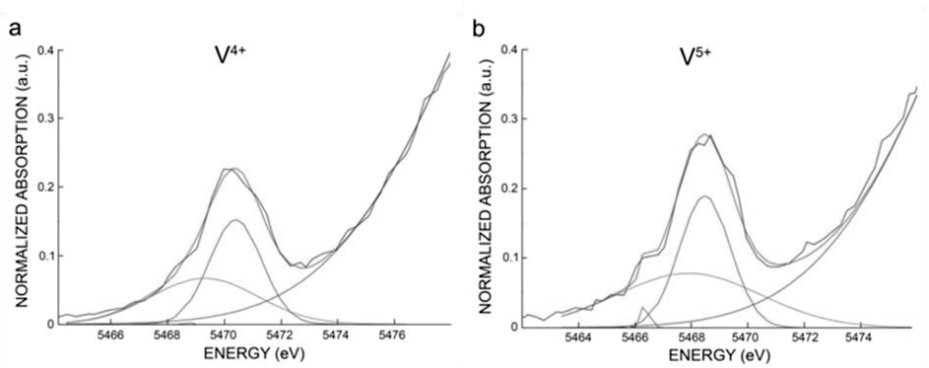

Figure 5. Simulation of vanadium pre-edge peak of vanadium species accumulated in mycelium of *P. blakesleeanus* upon addition of vanadyl sulfate (a) and sodium orthovanadate (b).

The second region of interest in XAFS spectra is the absorption edge which also depends on the oxidation state of examined element. For the elements whose atomic configurations do not imply the existence of d-orbitals, this is the main parameter which outlines the oxidation state. Thus, sulfur K-edge is sensitive to change in the valence of the element and per unit change in oxidation state approximately shift of 1.5 eV is common [4]. Although the absorption edge is shifted toward higher energies with increasing the valence of V and in some atoms play the crucial parameter in determination of oxidation state, these changes are often hardly visible (owing to an overlaps of its positions between several oxidation states) and except in the well resolved spectra, could not be considered as a reliable. However, the difference in the slopes of absorption edge curves of V compounds with various valences could be sometimes observed. It is particularly prominent in the case of the tetrahedral V^{5+} and some other V species (Figures 4 and 5). When the mixing of the various species is present, poor qualitatively information from the absorption edge features are accessible. The absorption edge region of XANES spectra culminates with the white line point. Intensity corresponding to that point is different from various samples and could not be dominant but rather a contributing factor in the resolving of V structure. White line is also may be considered as an onset of the post edge region which are shown to be very specific for the samples with a various structural characteristic [5,17,30]. Namely, the intensity of pre-edge peak of 5-valence V could often deviate from the expected high values which may indicates the lower oxidation state (Figure 4). Comparing the post-edge region with the suspected V^{4+} could serve as an efficient reason for the revealing of problem [30]. Of course, not only the intensity of pre-edge peak but also the shape of the peak has to be taken into account.

Conclusion

Speciation of V in biological systems deserves a great attention because of its multiple metabolic effects. Since the influence of various V species have a different effects in various organisms, cognition of their distribution and action in samples are necessary. XANES is the synchrotron-based spectroscopic technique which on efficient way determines crucial physical chemical features of all forms of accumulated V. It is particularly useful in revealing of the participation of various V species which could be accumulated in the same organisms. Therefore XANES represent the unique method in elucidation of crucial characteristics of V in living systems.

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Semi rational design of cellobiose dehydrogenase from *Phanerochaete chrysosporium* for increased oxidative stability

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Cellobiose dehydrogenase (CDH, EC 1.1.99.18) from *Phanerochaete chrysosporium* belongs to a group of oxidoreductases and has the ability to degrade different components of woody plants. CDH is secreted by wood degrading, phytopathogenic and saprotrophic fungi and this widespread appearance implies hers important function and makes her an important enzyme for applications in industrial and biotechnological processes, as well as biosensors and biofuel cells. Cellobiose dehydrogenase is also used in industry for bleaching cotton and in food industry for lactose detection. CDH is monomeric enzyme consisting of two domains, flavin domain containing FAD as cofactor and smaller hem *b* containing cytochrome domain, connected via flexible linker. Physiological role of CDH is reflected in the degradation of cellulose and lignin in cooperation with other cellulolytic enzymes, because CDH catalyzes oxidation of celobiose (Glc –β–1,4 Glc) and other β–1,4–linked disaccharides and oligosaccharides to the corresponding lactons. Enzymes used in biosensors and for bleaching cotton should have high stability, especially toward reactive oxygen species. In order to improve oxidative stability of CDH, we have mutated CDH and tested its stability in presence of hydrogen peroxide. After successful cloning of the CDH gene in pYES2 vector, saturation mutagenesis was used to make library mutants where tree methionine residues were mutated. Residual activity of mutants was measured after the enzyme incubation in 0.3 M hydrogen peroxide for 0, 2 and 6h. After analysis of large number of mutants, it was observed that three mutants are showing higher oxidative stability compared to the wild – type enzyme. Residual activities of these mutants after 6 hour incubation in the hydrogen peroxide were over 50%, whereas wild-type has 30%. Selected mutants were expressed in *S. cerevisiae* and purified on DEAE column. Purity and activity of the enzymes were detected on the electrophoresis gel, oxidative stability of purified mutants was measured once again and characterization of these mutants was done. Mutants showing increased oxidative stability were sequenced and we have decided to

combine these mutations with each other in order to make combined mutants that will be tested for oxidative stability.

Fractionation and characterization of lignans from *Anthriscus sylvestris* root extracts

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Anthriscus sylvestris (L.) Hoffm. (wild chervil) is a plant from *Apiaceae* family, nowadays widespread over the world. It has only a limited use as a food and as a medicinal plant (mostly in China, Japan and Korea) and it is not a part of the official medicine. Therefore, it is not cultivated and it is considered a noxious and invasive weed. However, the scarce reports on its chemical composition confirm the abundance of dibenzobutyrolactone and aryltetralin lignans structurally related to *Podophyllum* lignans, with extremely potent antiproliferative, antiinflammatory, fungicidal and insecticidal activity. Thus, *A. sylvestris* may represent a promising source of bioactive natural products, that could alleviate the unsustainable exploitation of *Podophyllum* species.

In this study, root extract of *A. sylvestris* was fractionated using liquid-liquid extraction, flash chromatography and centrifugal partition chromatography, and screened for lignans using LC-DAD-MS/MS and NMR, with a final goal of obtaining the neat compounds for structural characterization and bioactivity evaluation.

Air-dried roots of *A. sylvestris* specimens (492 g) were percolated with methanol (5 cycles). The raw extract (75.4 g) was purified by liquid-liquid extraction: partitioning in water-chloroform system removed bulk polar compounds (60.3 g), while subsequent partitioning in acetonitrile-hexane system removed some highly lipophylic components (1.50 g), yielding 13.5 g of lignan-enriched extract.

The enriched extract (8.92 g) was fractionated by automated flash chromatography on silica column. The fractions (defined by TLC, and labelled F1–F9) were eluted using hexane-acetone gradient. By using LC-DAD-MS and ^{1}H NMR, the presence of lignans in F6–F9 was confirmed. The most abundant fraction, F6, was found to consist mostly of well-known, major lignans, including yatein, nemerosin and deoxypodophyllotoxin, while the fractions F7 and F8 contained a number of unknown structures.

F6 (2.62 g) was further fractionated by CPC, using HEMWat-00 (hexane/ethyl acetate/methanol/water 1:1:1:1) as a solvent system, to obtain fractions F6.1–F6.9. Through separation of the dominant components, the fractionation allowed for a more comprehensive LC-MS analysis, thus resulting in detection of at least 24 different lignans (most of them, for the first time in *A. sylvestris*).

F7 (961 mg) was further fractionated by flash chromatography on silica column. Fractions, designated F7.1–F7.10, were eluted using hexane/ethyl acetate gradient. LC-MS confirmed the presence of at least 29 lignans (only 7 of which were previously found in this species).

Finally, F8 (466 mg) was also separated by flash chromatography, employing silica column and hexane/ethyl acetate gradient, to afford fractions F8.1–F8.7. Not less than 23 different lignans were detected by LC-MS, only 5 of which were previously reported in *A. sylvestris*.

To summarize, by combining preparative chromatography techniques and LC-MS analysis, over 30 unique lignan structures were detected in *A. sylvestris* root extract, thus confirming that this species is a a rich source of diverse lignans. The simplification of composition and removal of the major constituents allows for further separation by pHPLC, in order to isolate the pure compounds.

Directed evolution of cellobiose dehydrogenase for higher activity

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Cellobiose dehydrogenase (CDH) gene from *Phanerochaete chrysosporium* has been cloned in yeast *Saccharomyces cerevisiae* for extracellular expression. Recombinant CDH produced in yeast had lower specific activity of 0.6 U/mg than native CDH produced in *P.chrysosporium*. Recombinant enzyme showed similar substrate specificity for cellobiose and lactose. Optimal temperature and pH stability was slightly different compared to native CDH. The molecular weight of recombinant CDH was higher than molecular weight of native CDH (90 kDa) with a broad band on SDS electrophoresis gel at 120kDa that was result of hyperglycosylation. Results showed that CDH can be expressed in yeast *S. cerevisiae* that can be used in directed evolution experiments. CDH gene library was generated using error-prone PCR to create random mutations. Mutants were tested in microtiter plates for improved activity using adapted DCIP assay. Several mutants with increased activity were detected in microtiter plates and therefore purified and further characterized.

The gene for recombinant wild type cdh and one of the best mutants had been cloned in heterologous yeast *P.pastoris.* Mutant showed higher specific activity than rCDH. Temperature profiles were similar for both enzymes as well as pH optimum. Substrate specificity was similar in both enzymes with slightly higher Km for lactose in mutant than in rCDH. Molecular weight was similar to wtCDH from fungus (approximately 90 kDa). Obtained results showed higher productivity in *P.pastoris* and difference in specific activities between mutant and rCDH. We used *P. pastoris* for efficient production and characterization of enzymes.

Carbonylation of HSA with methylglyoxal leads to release of copper(II) ions and changes in its antioxidant capacity

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Human serum albumin (HSA), has important physiological functions like regulation of oncotic pressure and also serves as a transporter of metal ions, fatty acids, cholesterol, hormones. With normal serum concentration between 35 and 50 gL^{-1}, and 70–80% of its Cys34 in sulfhydryl form, HSA is the predominant serum protein and a major plasma antioxidant. It contains two binding sites for copper ions, one with high affinity, located at the N-terminus (NT).

The goal of this study was to decipher the effect of HSA modification with methylglyoxal (MG) on its ability to bind and sequester Cu^{2+}, which could prove to be useful in better understanding and possible treating of pathological states with prominent oxidative/carbonyl stress such as diabetes.

In this study, we monitored the changes in Cu^{2+} ion and Cys34-thiol group content, as well as their ratios, upon modification of HSA with MG. Also, the conformational changes in HSA molecule were monitored by recording fluorescent spectra. Electrophoretic properties of samples were monitored by performing SDS and native PAGE. Samples from diabetic patients and controls were also analyzed.

The ability of HSA to coordinate Cu^{2+} decreases upon carbonylation of the Cys34-SH group. Carbonylation of Cu–HSA complexes caused a decrease in Cys34-SH content and leakage of Cu^{2+} from Cu-HSA complexes. Conformational changes in samples modified with MG were also observed. The ratio between the percentage of reduction in the Cys34-SH group content and the percentage of release of Cu^{2+} from complexes is 2.12 ± 0.28. The same ratio (1.96 ± 0.36) was obtained upon oxidation of the Cys34-SH group (with no changes in HSA conformation), leading to conclusion that the binding/release of Cu^{2+} by HSA depends mainly on the redox state of the Cys34-SH group. Samples from diabetic patients had significantly lower contents of Cys34-SH and HSA-bound Cu^{2+} (0.457 ± 0.081 mol SH per mol HSA, 10.7 ± 0.01 mmol per mol HSA, resp.) ($p < 0.01$) (0.609 ± 0.027 mol SH per mol HSA; 13.4 ± 0.01 mmol per mol HSA, resp.). Strong correlations between the values for HSA-SH and glycated hemoglobin, HbA1c, ($R = -0.803$, $p < 0.01$), and between the values for the HSA-bound Cu^{2+} content and HSA-SH content ($R = 0.841$, $p < 0.002$) were found in the diabetic group.

Overall, the reaction of HSA carbonylation *in vitro* (with MG) and *in vivo*(diabetes), leads to release of Cu^{2+} ions from copper–HSA complexes in an extent which depends mainly on the redox state of the Cys34 free thiol group.

Investigation of vitamin C effects on DNA damage during enzymatic decolorization

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Introduction

DNA damage is partially mediated by reactive oxygen species (ROS) which can lead to mutations and development of cancer [1]. Complex azo colorants can be generators of endogenous ROS by: dye itself, during reductive biotransformation or by products obtained after oxidation [2]. Horseradish peroxidase (HRP) is an oxidoreductase which can catalyze degradation of numerous aromatic substrates, such as dye molecules, in the presence of hydrogen peroxide (H_2O_2) [3]. However, the effect of enzymatic decolorization should take into account the toxicity of degradation products as well as the percentage of color removed [4]. Since vitamin C is one of most studied antioxidants with demonstrated *in vitro* potential inprevention of oxidation of important biological molecules, we aimed to investigate the protective effects during enzymatic decolorization. We have applied medium throughput comet assay to measure DNA damage in lymphocytes of healthy adults treated with azo dyes before and after decolorization by HRP.

Materials and methods

Orange II (OR2, λ_{max} 490 nm) and Amido Black 10B (AB, λ_{max} 600 nm) were dissolved in 20 mM bicarbonate buffer pH 9.0 (BB). Vitamin C (L-ascorbic acid) was freshly prepared as stock 113 mM solution in water. Decolorization assay were done as follows. Dye solution of 100 μg mL^{-1} was incubated with 20 μL of 3 U ml^{-1} HRP and 170 μM H_2O_2. In parallel, decolorization reaction was prepared with addition of 50 μM vitamin C. Reaction mixtures were incubated 2 h at room temperature in the dark. Afterwards, decolorization of the dyes were calculated based on the formula: $D\ (\%) = [(A_i - A_d)/A_i] \times 100$, where A_i is the absorbance of the dye prior decolorization at λ_{max} and A_d is the absorbance after decolorization treatment.

Peripheral human lymphocytes were isolated from venous blood of healthy adults by centrifugation over Lymphoprep following manufacturer's instructions. The lymphocytes were embedded in 1% LMP agarose and treated with 30 μL of reaction mixture for 30 min.

Detection of DNA damage was done using the comet assay. Medium throughput comet assay was done by using the 12-gel comet chamber as described by Janovic et al. [4].

Results

HRP showed high efficiency in decolorization of OR2 and AB. In case of OR2 degradationwas 91% and vitamin C showed negligible effects on decolorization (90%). When AB was treated with HRP 72% of dye was decolorized. Interestingly, removal of AB in the presence of vitamin C was less efficient, where 7% less dye was removed under the same experimental conditions (Figure 1a).

Treatment with BB had no effects on lymphocytes, while some DNA damage was observed in the presence of vitamin C (Figure 1b). DNA damage was 32% and 21% for OR2 and AB, respectively. After the HRP treatment, DNA damage hasdecreased in both cases, but some percentage of tail DNA was present (Fig. 1c; HRP treated). This could be partially mediated by fast decolorization and residual H_2O_2 in the reaction mixture. Nevertheless, when the same decolorization reaction took place in the presence of vitamin C, DNA damage was slightly influenced (Figure 1c; HRP treated + vitamin C). These results indicate minor pro-oxidant effects of the vitamin C under the experimental conditions applied. Similar, but yet more pronounced, effects were observed in the case of double azo dye, AB, than the single azo dye OR2.

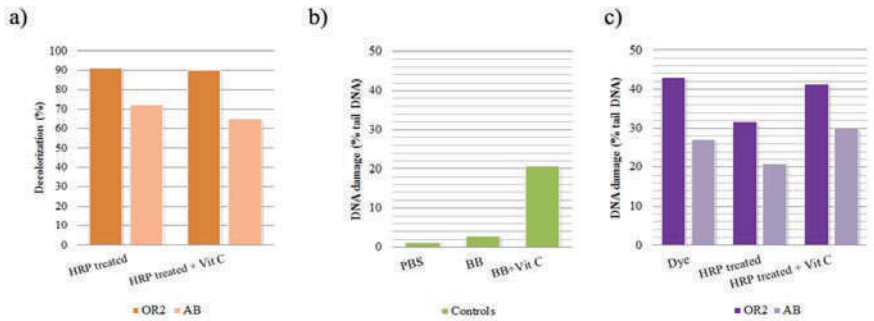

Figure 1. Effects of vitamin C (50 μM) on: a) decolorization of dyes by HRP; b) DNA damage in the negative controls (PBS – phosphate buffered saline solution, BB – bicarbonate buffer); c) DNA damage before and after HRP decolorization.

Conclusions

Using the comet assay we have investigated the possible protective effect of vitamin C against DNA damage caused by excess or remaining H_2O_2during or after decolorization of azo dyes. The potential of HRP for dye decolorization was showed in terms of reduction of genotoxicity potential of dyes tested. Vitamin C showed no effects in protecting DNA against potential damage occurring during oxidoreductase reaction.

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Molecular mechanisms of redox status and antitumor activity of extracts of invasive plant species (*Robinia pseudoacacia* and *Amorpha fructicosa*) in MRC-5 and MDA-MB-231 cell lines

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Breast cancer is a complex disease, and is one of the most common malignant diseases in female population. In recent years there is a great interest in prevention and treatment of malignant diseases by using of drugs derived from nature. An essential aspect in the strategy of cancer treatment and design of anticancer drugs is their ability to lead to the death of cancer cells and inhibition of their invasive potential.

The aim of this study is *in vitro* investigation of antitumor activity of methanol extracts of invasive plants *Robinia pseudoacacia* and *Amorpha fructicosa* on the basis of molecular mechanisms of redox status of immortalized cell lines of healthy lung fibroblasts (MRC-5) and breast cancer (MDA-MB-231).

Evaluation of antitumor effects of tested plant extracts is carried out on the basis of the following parameters: cytotoxic activity (determined by MTT assay), antiinvasive potential (determined by examining concentration of MMP-9 [1]), concentration of reactive species (superoxide anion radical, $O_2^{\bullet-}$ by NBT assay, nitrites by Griess method) and concentration of reduced glutathione (GSH). The effects of treatment on mRNA expression of *HIF-1α* and *iNOS* genes were determined by PCR method [2]. Methodology of determination of cytotoxicity, $O_2^{\bullet-}$, nitrites and GSH were previously described in brief [3].

Results showed that methanol extracts significantly reduced viability on MRC-5 and MDA-MB-231 cells, but the MRC-5 cells are more sensitive to treatment. According to the obtained results cytotoxicity is not significant (IC_{50} > 500 μg/ml, except for *A. fructicosa* on MRC-5 cells). The basal concentration of MMR-9, as parameter of invasive cell potential, in MRC-5 cells was 12.5 times lower than in MDA-MB-231 cells, which confirm invasive potential of the cancer cells. *A. fruticosa* at dose of 100 μg/ml significantly increased the level of MMP-9 in the MRC-5 cells, while both extracts significantly decreased MMP-9 level in MDA-MB/231 cells (Figure 1), indicating antiinvasive potential of treatments in cancer cell line. Investigated extracts did not change, or slightly lowered levels of $O_2^{\bullet-}$ in both cell lines, except in treatment with *R. pseudoacacia* in MDA-MB-231 cells, which induced prooxidant effect. Extracts of both

plants significantly reduced the level of nitrites and increased level of GSH in both treated cell lines. Also, we investigated gene expression of two key proteins of redox status. Extracts of both tested plants reduced level of mRNA expression of *HIF-1α* gene in MRC-5 cells, and significantly stronger effects are displayed in MDA-MB-231 cells (Table 1). These results directly correlated with decrease of MMP-9 concentration and antiinvasive potential of plants extracts. *R. pseudoacacia* and *A. fruticosa* significantly changed the expression of mRNA for *iNOS* gene in both tested cell lines (Table 1) and changes correlate with nitrite concentration measured after treatments.

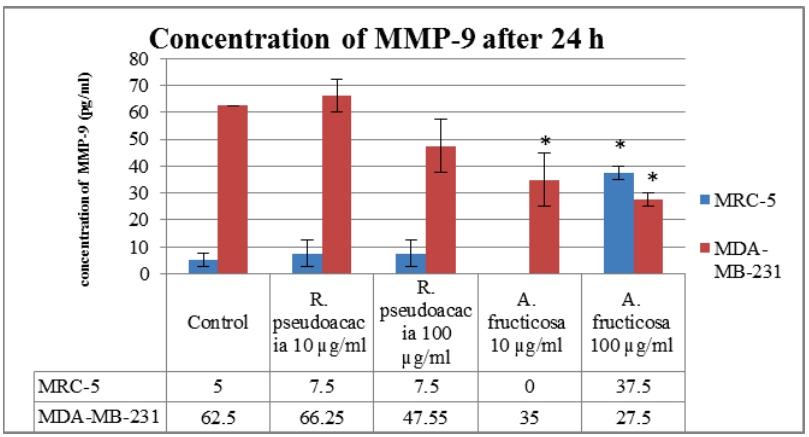

Figure 1. Effects of *R. pseudoacacia* and *A. fruticosa* methanol extracts on MMP-9 concentration in MRC-5 and MDA-MB-231 cell lines 24 h from treatment. Results are expressed as means ± SE. *p < 0.05 compared to control.

Table 1. Effects of *R. pseudoacacia* and *A. fruticosa* methanol extracts on expression of mRNA for *HIF-1α* and *iNOS* genes in MRC-5 and MDA-MB-231 cell lines 24 h from treatment.

Expression of mRNA of *HIF-1α* gene					
Cell line	**Control**	***R. pseudoacacia* 10 µg/ml**	***R. pseudoacacia* 100 µg/ml**	***A. fructicosa* 10 µg/ml**	***A. fructicosa* 100 µg/ml**
MRC-5	0.86 ± 0.03	0.79 ± 0.01	0.81 ± 0.04	0.75 ± 0.01	0.79 ± 0.04
MDA-MB-231	0.87 ± 0.02	0.41 ± 0.01*	0.76 ± 0.01*	0.54 ± 0.01*	0.77 ± 0.02*
Expression of mRNA of *iNOS* gene					
MRC-5	0.86 ± 0.01	1.11 ± 0.035*	0.77 ± 0.01*	0.85 ± 0.01	0.68 ± 0.01*
MDA-MB-231	0.79 ± 0.02	0.81 ± 0.01	0.85 ± 0.04*	0.84 ± 0.04*	0.65 ± 0.01*

Results are expressed as means ± SE for two independent determinations. *p < 0.05 compared to control.

In conclusion, both plants have shown significant antitumor effects. The effects of *A. fructicosa* are stronger - better cytotoxicity on MRC-5 cells, and the anti-invasive potential was better in MDA-MB-231 cells. Both plants significantly changed the concentration and gene expression of tested parameters of redox status. More significant effects showed *A. fructicosa*, and the changes are more pronounced in MDA-MB-231 cells.

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Effect of ellagitannins on oxidative stress of colorectal adenocarcinoma cells Caco-2

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The pomegranate tree (*Punica granatum* L.) is a deciduous fruit bearing small shrub from the *Lythraceae* family. Pomegranate tree has been cultivated for centuries in Eastern and Middle Eastern countries and due to its beneficial properties has been extensively used in folk medicine [1]. The results of numerous studies indicate that pomegranate may be effective in the treatment of obesity, inflammation, diabetes and the regulation of blood lipid parameters, and hence metabolic syndrome [2]. The health-promoting features are mainly attributed to the high content of total polyphenolic compounds, especially ellagitannins (ETs). Ellagic acid (EA) is a highly bioactive compound and represent hydrolysate of ellagitannins [2]. ETs are hydrolysed to ellagic acid which is metabolised in colon to urolithin-A (UROA; 3,8-dihydroxy-6H-benzo[c]chromen-6-one), urolithin-B (UROB; 3-hydroxy-6H-benzo[c]chromen-6-one), urolithin-C (UROC; 3,8,9-trihydroxybenzo[c]chromen-6-one), urolithin-D (UROD; 3,4,8,9-tetrahydroxybenzo[c]chromen-6-one), urolithin-A glucuronide (UROAG; 3,8-dihydroxy-6H-benzo[c]chromen-6-one glucuronide) and urolithin-B glucuronide (UROBG; 3-hydroxy-6H-benzo[c]chromen-6-one glucuronide) [3,4]. EA, UROA and UROB, at concentrations achievable in the lumen from the diet, might contribute to the colon cancer prevention by modulating the expression of multiple genes in epithelial cells lining the colon [5]. The aim of this work was to assess the effect of ETs on the growth and oxidative status of colon epithelium using as a model colorectal adenocarcinoma cell line (Caco-2).

The human colon cancer cell line Caco-2 were grown in the presence of EA metabolites (UROA, B, C, D, and a mixture of all four). The cell vitality was assessed by measuring the release of lactatate dehydrogenase (LDH) in the cell culture supernatant. Trypan blue dye exclusion test was used to assess the cell viability before immediate stimulation experiments [6]. The level of intracellular reactive oxygen species was assessed by measuring the oxidation of the probe 2′, 7′-dichlorofluorescin diacetate [7]. Caco2 cells were treated with UROs. The effects of short term stimulation and growth in the presence of urolithins were measures via flow cytometry. Antioxidative action was further investigated

by measuring the activity of oxidative stress enzymes catalase, superoxide dismutase and glutathione peroxidise using commercial kits (Cayman Chemicals).

Statistically significant effects on cell vitality were noted for UROA and UROB at 300 μM, while lower concentrations had no detectable effect on the LDH activity in the supernatant. In the case of UROC and UROD cells, there was no visible cytotoxicity. The concentrations of UROA and UROB that affected cell viability were 10 times higher that reached in the gut lumen after consumption of pomegranate juice. A mixture of UROs did not exert any significant effect on cell vitality. The potential protective effect of URO on scavenging intracellular reactive oxygen species (ROS) was evaluated through two types of treatment: a long term pre-incubation condition that might reflect a long term preventive action and a short term incubation treatment which may represent a possible therapeutic administration. The Caco-2 cells were challenged with H_2O_2-induced stress and pre-incubated with URO. URO was shown to reduce the generation of H_2O_2-induced radicals. In the short-term incubation state the reduction was more pronounced.

Effects of URO was further investigated by measuring the activity of oxidative stress enzymes catalase, superoxide dismutase (SOD) and glutathione peroxidase (GPX). Supplementation of growth medium with URO affected significantly the activity of catalase as it was decreased in the treated cell lysate. SOD and GPX activity in the cell lysate was unaffected by the growth in the presence of URO mixture.

Evidence from *in vitro* and *in vivo* laboratory studies, clinical trials and epidemiological investigations show that plant-based diets have protective effect against various cancers. The antiproliferative effect of plant polyphenolics has been clearly demonstrated *in vitro* and in animal models, which suggest that consumption of these compounds with in the diet can may have a preventive role against cancer development [5].

Previous reports have found that EA inhibits growth of colon cancer cell at concentrations bellow 100 μM [8]. The inhibitory effect of URO on Caco-2 cell growth was noted only at 300 μM concentration and only for UROA and UROB. Concentrations bellow 300 μM did not show any significant effect on cell vitality. In glycosylated form they are less potent in inhibiting the growth of Caco-2 cells and this is probably due to the fact that glycosylated form is less lipophilic and as such is less likely to penetrate the cells then the unglycosylated form.

Under the experimental setup we employed in our investigation we were not able to detect any significant cytotoxicity of the URO mixtures used. Ellagic acid itself has a more potent antiproliferative effect then its metabolite and the lack of EA in the mixture we used can explain why we noted almost no cytotoxic effect with the mixtures we used. The primary antioxidant mechanism of EA has been attributed to the direct scavenging of free radicals, nitrogen reactive species, and ROS, including hydroxyl radicals, peroxyl radicals, NO_2 radicals, and peroxynitrite.

Short term exposure of cells to URO can lead to a significant decrease of ROS in the cells thus exerting preventive action against ROS induced cell damage. Furthermore, UROs can modulate the activity of antioxidative enzymes, catalase at least. It is important to note that the chemoprotective action of URO is not limited merely to ROS scavenging but also to

modulation of enzyme activity. At concentrations reached in the lumen of the gut, urolithins can exert beneficial effects on the cells by decreasing oxidative stress within the cells thus preventing the damage caused by reactive oxygen species. In such a way UROs can ameliorate if not prevent the damage that is caused by ROS with the cell helping with the prevention of oxidative stress related pathologies.

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Chemical characterisation and biological potency of merlot variety grape juice and wine from Fruška Gora region

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Numerous reports confirm positive effect of grapes, grape juice and wine in the treatment of various disorders, such as cardiovascular and neurodegenerative diseases, ischemic stroke, cancer and ageing. The region of Fruška Gora, mountain in the Northern part of Serbia, is famous for the old winemaking tradition. However, there is nearly no data about detailed phytochemical profile or biological potential of wines from Fruška Gora region.

The aim of this study was to comprehensively examine polyphenolic profile and biological activities (neuroprotective and antioxidant) of Merlot wine from Fruška Gora, which, to the best of our knowledge, have never been investigated before. Also, related grape products, very popular local beverages, such as grape juice (juice obtained by immediate pressing of crushed grapes) and young wine (first wine obtained after fermentation), were covered by this study in order to evaluate their characteristics and monitor change in chemical composition and biological activities during aging of wine.

An LC-MS/MS technique was applied to evaluate the quantitative content of 47 polyphenolics, including 16 phenolic acids, 26 flavonoids, 3 coumarins and 2 lignans, followed by HPLC-UV/VIS technique that detected 5 anthocyanins. Antioxidant potential was evaluated using spectrophotometric tests, based on measuring nitric oxide and diphenylpicrylhydrazyl (DPPH) radical scavenging activity, potential of lipid peroxidation inhibition and reducing power (FRAP assay). Neuroprotective effect was estimated through inhibitory potential towards acetylcholinesterase (AChE), a key enzyme catalysing the hydrolysis of acetylcholine in the nervous system. AChE inhibitors are used in treatment of many neurological diseases, such as Alzheimer's disease.

LC-MS/MS analyses of selected polyphenols resulted in detection of 30 of 52 compounds and, in general, the lowest polyphenolic content was found in grape juice. Among analized phenolic compound, catechin was dominant flavonoid in Merlot juice, young wine and wine (7.38, 7.70 and 6.84 mg/L, respectively), followed by myrcetin (1.10, 1.20 and 1.25 mg/L). Ellagic acid was the most frequent phenolic acid (0.76, 1.10, 3.16 mg/L), while malvidin-3-*O*-glucoside was the dominant anthocyanin in all samples (24.0, 79.0, 57.0

mg/L). The content of resveratrol, a stilben compound whose main dietary source are grape seeds and skin, and consequently the wine, was increasing from 0.80 in Merlot juice to 2.42 mg/L in wine. The Merlot young wine exibited the best potential of DPPH neutralization, while in all other antioxidant assays wine expressed the highest activity. Considering neuroprotective activity, grape juice evinced the greatest potential of AChE inhibition.

Since polyphenolic profile is used for authentication, classification of wines of a certain variety, geographical region, manufacturing process, vintage, prediction of sensory properties etc., while antioxidant activity is one of the main health benefits of wines, obtained results contribute to overall characterization of Merlot wine and juice from Fruška Gora region. Moreover, they additionally support suggestion to consume non-alcoholic grape derivative, grape juice, as a rich source of health-beneficial compounds, such as natural antioxidant and neuroprotective agents.

Neuropeptide Y induces nitrosative stress and inhibits migration in human choriocarcinoma cell line JEG 3

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Neuropeptide Y (NPY) is a neuropeptide present in significant concentration in peripheral and central nervous system, and has a role in various processes such as anxiety, stress-related behavior, food intake, digestion, metabolism, immune response, memory and learning [1]. The migration of trophoblast cells is an essential step during placenta formation. Inadequate migration of trophoblasts results in poor placental perfusion and placental hypoxia/reoxygenation, which could result in development of oxidative stress that contributes to endothelial dysfunction and clinical manifestation of several pregnancy complications [2]. Since the levels of NPY are significantly higher in circulation of the women with preeclampsia in comparison to healthy pregnant women, the aim of this study was to investigate the potential effects of NPY on migration capacity of trophoblasts [3]. In this experiment we have also investigated the effects of NPY on intracellular nitrites production as one of the component of nitrosative stress which could be in correlation with cell migration capacity. Human choriocarcinoma cells were cultivated in cell culture medium (DMEM) in incubator at 37°C, in normal atmosphere containing 5% CO_2. NPY was applied in short-term (24 h) and long-term (72 h) treatments in highest non-cytotoxic concentration of 10 nmol/L, corresponding to its level in the blood of women with pregnancy complications. The levels of nitrites (NO_2^-) were determined spectrophotometrically, while the cell migration index was estimated by using Boyden chamber transwell assay [4,5]. The results of our study showed that the 1 nM NPY significantly decreased the production nitrites, an indicator of nitric oxide (NO) [6]. NO is multiacting signal molecule which could have a role in cell migration by mediating the phosphorylation state of cadherines, a cell adhesion molecules on cell membrane [7]. The decrease in NO concentrations that is induced by NPY could be the cause of diminished migration potential of trophoblasts. Overall, the results of our study showed that NPY may contribute to inhibition of trophoblast cell migration by decreasing the bioavailability of nitric oxide, potentially leading to a pathogenesis of various pregnancy disorders.

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Biodegradation of n-alkanes and polycyclic aromatic hydrocarbons by *Pseudomonas aeruginosa* san ai

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Alkanes and polycyclic aromatic hydrocarbons (PAHs) have been threatening the global environment because of their toxicity and low biodegradability. On the other side, the ability of *Pseudomonas aeruginosa* to grow in diverse environments, on different sources of carbon implies its potential for remediation of hardly biodegradable hydrocarbons with various chain length and structures [1,2]. Thus, in this study the *Pseudomonas aeruginosa* san ai, strain isolated from industrial alkaline mineral metal-cutting oil, as its natural habitat was investigated for its capability to degrade several n-alkanes and PAHs [3].

Pseudomonas aeruginosa san ai was cultivated for 10 days under aerobic conditions in liquid mineral salt medium supplemented with n-alkanes: n- hexadecane and n-nonadecane and PAHs: fluorene, phenanthrene and pyrene, respectively as a sole source of carbon and energy. The final concentration of carbon source was 2 mg/L. Samples were collected every day and analyzed for cell growth and biodegradation efficiency. The residual hydrocarbons were extracted and quantified by gas chromatography–mass spectrometry.

Strain *Pseudomonas aeruginosa* san ai grew rapidly with n-alkanes, showing degradation degree (in 10 days) of 98% and 95%, for n-hexadecane and n-nonadecane, respectively. It grew a bit slower when utilizing polycyclic aromatic hydrocarbons, signifying that PAHs are much more resistant to biodegradation. In spite of slower growth of culture, degradation of PAHs by *Pseudomonas aeruginosa* san ai was remarkable. Fluorene, phenanthrene and pyrene were degraded, respectively, by 100%, 90%, and 30% in10 days. Compared to PAHs of three aromatic rings, pyrene which contains four aromatic rings was more resistant to biodegradation.

The obtained results clearly shows that the *Pseudomonas aeruginosa* san ai can utilize n-alkanes and polycyclic aromatic hydrocarbons as carbon sources for growth. Undoubtedly, this strain is a potential candidate for the environmental bioremediation of hydrocarbon-contaminated area.

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In vivo toxicity of naked and coated CeO_2 nanoparticles

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The use of nanomaterials in various commercial products and industrial processes has increased. Although the application of nanoparticles has great importance, some of them can be risky to human health and the environment. Cerium oxide nanoparticles CeO_2 have been extensively investigated due to the excellent oxygen storage capacities on the basis of the redox transition between Ce^{3+} and Ce^{4+} and formation of oxygen vacancies on their surface. The effect of CeO_2 on individual organisms and the ecosystem in general are not sufficiently explored.

In this research we used CeO_2, naked and coated with three different carbohydrates (glucose, pullulan or levan), to studytheir effectin three different model systems. We analyzed bioluminescence in gram-negative bacterium *Vibrio fischeri*, and acute toxicity incrustacean *Daphnia magna* and zebrafish *Danio rerio*. In all experiments the concentration of CeO_2 nanoparticles was 200 mg/L. For all used types of nanoparticles, we observed bioluminescence inhibitionof around 20%. The mortality rate oftreated *D. magna* was 6.7% for glucose coated CeO_2, 9.2% for naked and pullulan coated CeO_2 and 18.2% for levan coated CeO_2. Despite the adherence of nanoparticles aggregates to the outer surface of the chorion, no acute toxicity was observed for zebrafish embryos during the first 72 h post fertilization. We also did not observe increased level of abnormalities among treated embryos during the first 72 h post fertilization.

In this study, no toxic effects of CeO_2 nanoparticles were observed, but the extent of uptake of nanoparticles remains to be investigated. The effect of chronic exposure to CeO_2 should also be analyzed.

Phycocyanobilin and chromopeptides from C-phycocyanin: structure, biological activity and binding to human serum albumin

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Spirulina (Arthrospira), photosynthetic, filamentous cyanobacteria, has been used as food for centuries. It is the richest known natural source of proteins and essential amino acids, excellent source of vitamins, macro- and micro-elements, essential fatty acids, glycolipids, and sulfated polysaccharides. C-phycocyanin (C-PC), the most abundant protein of *Spirulina*, is highly fluorescent and water soluble heterodimeric protein. Its blue color arises from covalently attached (*via* tioether bond) tetrapyrrole chromophore phycocyanobilin (PCB). One PCB molecule is attached to α-subunit *via* cysteine 84, while β-subunit binds two molecules of PCB *via* cysteines 82 and 153. Numerous studies have shown C-PC exhibit significant antioxidative and free radical-scavenging properties, anti-inflammatory, anti-platelet, anti-cancer and hepatoprotective effects. Furthermore, PCB itself exhibits various strong health-promoting activities. There are no literature data evaluating structure and bioactivities of released peptides with bound chromophore (chromopeptides) in C-PC digest, nor information on the PCB binding to human serum albumin (HSA), major plasma protein.

We examined the structures and bioactivities of chromopeptides obtained by 24 hours pepsin digestion of C-PC isolated from commercial *Arthrospira platensis* powder. SDS-PAGE under reducing conditions (16% gel) has shown that chromoprotein is rapidly digested by pepsin in simulated gastric fluid. HPLC (RP C-18 column) analyses of digest revealed five chromopeptide fractions. The structure of released chromopeptides was analyzed by high resolution tandem mass spectrometry and peptides varying in size from 2 to 13 amino acid residues were identified in both subunits of C-PC. It was shown that all chromopeptide fractions have significant antioxidant (ORAC and reducing power test) and Cu^{2+}-chelating (fluorescence quenching study) activities and show cytotoxic effect (MTT viability assay) on human cervical adenocarcinoma and epithelial colonic cancer cell lines. In addition, chromopeptides protect human erythrocytes from free radical-induced hemolysis in antioxidative capacity dependent manner. Digestion by pepsin releases biologically active chromopeptides from C-phycocyanin whose activity is mostly related to the antioxidative potency provided by chromophore.

Based on a computational approach (molecular modeling), we further demonstrated two putative high-affinity binding pockets on HSA for PCB chromophore. Predicted binding

sites were identical to the two previously identified binding sites for structural analog bilirubin (subdomains IB and IIA). Results obtained by protein and pigment fluorescence measurements, circular dichroism (CD), and fluorescence competition experiments confirmed high affinity (binding constant of 2.2×10^6 M^{-1} at 25ºC), stereoselective binding of PCB *M*-conformer to HSA, and its competition with bilirubin, warfarin (IIA site marker) and hemin (IB site marker). Our experimental data confirm that PCB binds to IB and IIA binding site of HSA with an affinity similar to bilirubin. Spectrophotometric titration experiments also confirmed stoichiometry of PBC binding to HSA (n = 2). In conditions characterized by an increased bilirubin plasma concentration, or intake of drugs binding to IB or IIA binding site, pharmacokinetics of PCB may also be changed. Furthermore, binding of PCB to HSA induces structural changes in the protein and in the ligand. UV-visible absorbance measurements indicate that bound PCB has more stretched conformation in comparison to free form. Experimental data have shown increase in thermal (CD and fluorescence melting curves data) and proteolytic stability (trypsin digestion study data), as well as the α-helical content (CD and FT-IR spectra data) of the protein upon ligand binding. Therefore, obtained results directly imply that binding of PCB to HSA may provide higher protein's stability and longevity in plasma.

Taken together, these results give new insights into bioavailability, distribution and nutraceutical properties of C-PC and/or its PCB chromophore of fundamental significance and applied potential.

Antiproliferative, antioxidative and promigratory effects of 3-benzyl-5-isoprpyl-5-phenylhydantoin in human breast cancer cell line MDA-MB-231

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Hydantoin and its derivatives are widely used as anticonvulsants for the treatment of epilepsy, cardiac arrhythmias and, more recently, tumors [1]. Cancer is one of the most lethal diseases of modern age, so the majority of studies is focused on the research of hydantoin derivatives as potential anticancer drugs [2]. In this study anti-proliferative, antioxidative, and migratory effects of hydantoin derivate 3-benzyl-5-isoprpyl-5-phenylhydantoin were tested on human breast cancer cell line, MDA-MB-231. The cells were cultivated in a cell culture medium (DMEM) in incubator at 37°C and in 5% CO_2 atmosphere. The cells were exposed to an increasing concentrations of 3-benzyl-5-isoprpyl-5-phenylhydantoin at values 0.01, 0.1, 1, 10, 50, and 100 μM. Anti-proliferative and antioxidant effects of this hydantoin derivate against MDA-MB-231 cell line were determined spectrophotometrically after short- (24 h) and long-term (72 h) treatments [3–5]. The effects on migration potential were tested for concentrations of 1 μM and 10 μM after 24 h and 72 h exposure. Migration index was determined by Boyden chamber transwell migration assay [6]. Levels of cytotoxicity appeared to be dose-dependent until the concentration of 10 μM when there have been no furher changes at increasing the concentration. The results obtained in this study showed that all used concentrations of 3-benzyl-5-isoprpyl-5-phenylhydantoin exhibited cytotoxic effects on MDA-MB-231 cells, which means that this hydantoin derivate exibits strong antiproliferative activity. The results also showed increased concentrations of nitrites at supernatants of cultivated cells, which indicate significant antixidative role of 3-benzyl-5-isoprpyl-5-phenylhydantoin. Elevated levels on nitrites indicate high levels of nitric oxide (NO) production. NO is a multiacting signal molecule which has a role in cell migration by mediating the phosphorylation of some cell adhesion molecules [7]. These data indicate that elevated NO production could be one of the causes of increased migration of this cell line induced by hydantoin derivate. Based on these results, it can be concluded that the tested hydantoin derivate acts as antioxidants by

increasing nitrites levels, has a strong antiproliferative activity and elevates the migration ratio of MDA-MB231 cell line. Taken together, these results indicate complex roles that 3-benzyl-5-isoprpyl-5-phenylhydantoin could have in its potential antitumor applications. Strong antiproliferative activity of this molecule also suggest its potential antitumor role, while on the other hand its promigratory effect on MDA-MB-231 cell line could mean that these hydantoin derivate may eventually promote metastatic potential of some cancer cell types. However, these data indicates that the roles of hydantoin and its derivatives as anticancer drugs are multiple and complex, and should be much more elucidated.

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Cytotoxic and antioxidative effects of Oligo-Grapes product and ethanolic extracts of pomace and red wine on HCT-116 and SW 480 cell lines

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Grapes and their products, such as wine and pomace, are containing high concentration of polyphenolic compounds [1]. Grape polyphenols exert many biological activities such as antioxidant, antinflammatory and anticancerogenic effects [2].

The aim of this study was to determinate cytotoxic and antioxidative effects of Oligo-Grapes product and ethanolic extracts of pomace and wine on colon cancer HCT-116 and SW 480 cell lines. Oligo-Grapes is a product of Bionys plus, Krnjevo, Serbia. The lyophilized pomace and red wine were extracted by soaking with ethanol, than filtered through filter paper and evaporated to dryness on a rotary evaporator at 40°C. HCT-116 and SW 480 cell lines were treated with various doses of Oligo-Grapes (OG), ethanol extracts of pomace (PE) and wine (WE) (dissolved in 0.5% DMSO) for 24 and 72 h. Evaluation of cytotoxic and antioxidative effects is carried out on the basis of the following parameters: cytotoxic activity (determined by MTT assay), concentration of reactive species (superoxide anion radical, $O_2^{\bullet-}$ by NBT assay, nitrites by Griess method), concentration of reduced glutathione (GSH) and protein expression of inducible nitric oxide synthase (iNOS) by immunofluorescence method. The used methodology was previously described in brief [3].

Our results showed that OG, PE and WE significantly decreased HCT-116 cell viability in dose-dependent way, while only in low doses treatments decreased SW-480 viability. However, cytotoxicity is not significatnt, when expressed as IC_{50} values (doses of treatment that inhibits 50% of cell growth). When discussed redox status parameters, all followed treatments significantly decreased of superoxide anion radical levels in both treated cancer cell lines (Figure 1), indicating significant antioxidative potential of tested grape preparations. The concentration of nitrites significantly increased in HCT-116 and SW-480 cells in the presence OG, PE and WE on dose- and time-dependent way (Figure 2), which are consequence of increased iNOS protein expression in colon cancer cells. The concentration of GSH in HCT-116 cells significantly increased after 24 h of exposure to OG, PE and WE, while with prolongated treatment period (72 h) GSH level decreased. In SW-480 cells this parameter significantly decreased after followed treatments.

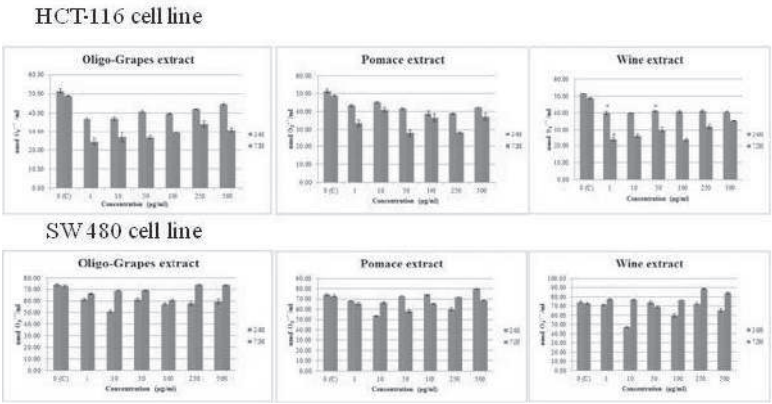

Figure 1. The effects of Oligo-Grapes, pomace and wine extract on superoxide anion radical level in HCT-116 and SW480 cells, after 24 and 72 h (left and right column, in each pair, respectively) exposure. Results were expressed as the means ± SE from 3 independent determinations. *statistically significant difference ($p < 0.05$) compared to control.

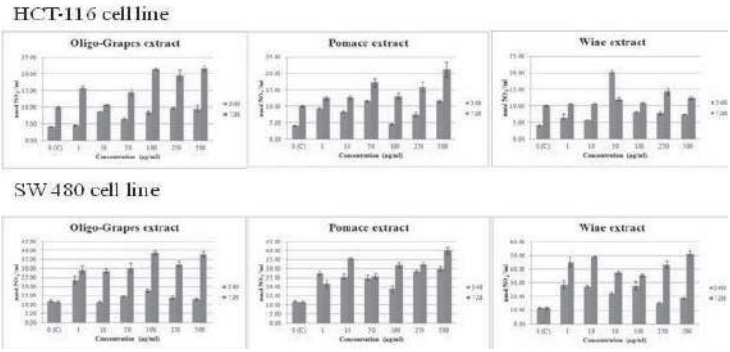

Figure 2. The effects of Oligo-Grapes, pomace and wine extract on nitrite concentrations in HCT-116 and SW480 cells, after 24 and 72 h (left and right column, in each pair, respectively) exposure. Results were expressed as the means ± SE from 3 independent determinations. *statistically significant difference ($p < 0.05$) compared to control.

In conclusion, tested Oligo-Grapes product and extracts of pomace and wine decreased of colon cancer cells viabilities, with stronger effects on HCT-116 cells, but without significant cytotoxicity. The treatment showed antioxidative effects, represents as superoxide anion radical level decrease. Increased iNOS protein expression and nitrite concentration in HCT-116 and SW-480 cells after treatments, and implication of these results in possible molecular signaling of grapes metabolism and nitrosative effects will discussed.

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Fenitrothion-induced changes of haemato-biochemical and oxidative stress parameters in rat blood: Protective role of selenium and vitamin C

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Organophosphate insecticides represent one of the most widely used classes of pesticides with high potential for human exposure in both rural and residential environments [1]. Fenitrothion [O,O-dimethyl-O-(3-methyl-4-nitrophenyl) phosphorothioate] (FNT) is a broad-spectrum organophosphate insecticide that distresses nervous system by inhibiting acetyl cholinesterase activity [1,2]. It is employed in agriculture to control insects and mites, as well as fly, mosquito and cockroach residual contact spray for farms and public health programs [1,3]. Fenitrothion residue is found primarily in soil, water and food products and can lead to a variety of toxic effects on the immune, hepatobiliary and hematological systems [3,4]. Red blood cells (RBC) are highly susceptible to oxidative damage due to the presence of heme iron, PUFA and oxygen radicals that may initiate the reactions which induce oxidative changes [5]. Antioxidants such as vitamin E, vitamin C (Vit C), alpha lipoic acid, selenium and melatonin possess efficacy in reducing lipid peroxidation in biological systems by inhibiting the formation of free radicals [6,7]. The aim of this study was to investigate the possible protective effects of selenium (Se, 0.5 mg/kg b.w, as Na_2SeO_3) and Vit C (100 mg/kg b.w) on altered hematological, biochemical and oxidative stress parameters in the blood of rats treated with FNT (20 mg/kg b.w) for 30 days. Exposure of rats to FNT caused changes of some haematological parameters (RBC count, Hb concentration and Ht value), suggesting that the FNT induced haematotoxicity. FNT reduced serum total protein and albumin, while plasma transaminases (ALT and AST), lactate dehydrogenase activities and bilirubin levels increased. FNT induced oxidative stress and altered the glutathione redox status (GSH and GSSG), leading to an increased concentrations of lactoperoxidase in RBC of rat. Intoxication of rats with FNT was followed by decreased activity of antioxidant enzymes (SOD, CAT and GSH-Px). Coadministration of selenium and Vit C reduced lipid peroxidation and restored the levels of examined haematological, biochemical and oxidative stress parameters, as well as the levels of AOS to their near-normal levels when compared to FNT-treated rats.

In conclusion, the results suggest that FNT has significant prooxidative effects and may disrupt oxidant/antioxidant balance in erythrocytes. Furthermore, the combination of selenium and Vit C can reduce the toxic effects of FNT in erythrocytes of rats.

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Phenolic profile and antioxidant potential of grape vine leaves

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Fresh leaves of the grape vine are mostly consumed in the diet of the Mediterranean and Asian countries, and also in Serbia. Thanks to their beneficial effects, which have been recognized since ancient times, the extracts of dried leaves are traditionally used in folk medicine for treating a wide range of health conditions. The leaf extracts are usually applied in cases such as hemorrhage, hypertension, diarrhea, eye infections, diabetes and circulatory system and inflammatory disorders.

Unlike other parts of the grape vine that are extensively investigated, there is still not enough data on the chemical composition and biological activity of *Vitis vinifera* leaves. Therefore, the aim of this study was to evaluate the antioxidant activity and determine the phenolic profile of three grape vine leaf varieties. Leaf extracts of red (Cabernet Sauvignon, Muscat Hamburg) and white (Italian Riesling) grape varieties, grown in the vineyards of Fruška Gora, Serbia, were prepared by the extraction of air-dried leaves with 80% methanol. Quantitative analysis of 45 phenolic compounds was performed by the LC-MS/MS technique. Antioxidant potential was evaluated with standard spectrophotometric tests by measuring the reducing power (FRAP assay) and free radical scavenging ability of the samples towards diphenylpicrylhydrazyl ($DPPH^{\bullet}$), superoxide anion ($O_2^{\bullet-}$) and hydroxyl ($HO^{\bullet}$) radicals.

Forty five phenolic compounds were examined while 21 were found in leaf extracts, among which quercetin–3–*O*–glucoside was the most abundant flavonoid in all samples (Cabernet Sauvignon: 4.41 mg/g; Muscat Hamburg: 6.18 mg/g; Italian Riesling: 3.85 mg/g). Cabernet Sauvignon grape vine leaves expressed the highest scavenging capacity of all three free radicals ($DPPH^{\bullet}$, $O_2^{\bullet-}$, $HO^{\bullet}$), while the extract of Muscat Hamburg showed the best reducing ability. Compared to a synthetic antioxidant – propyl gallate, all samples exhibited good activity.

In this research novel data on Serbian grape vine leaf varieties are introduced, including a detailed phenolic composition and information on the antioxidant potential. The leaves represent a rich and affordable source of phenolic compounds with good therapeutic potential. Therefore, the results support the use of *V. vinifera* leaves in diet, as well as their

utilization that could aid the food and pharmaceutical industry and solve ecological issues concerning waste management.

Structural insight into the myotilin-actin interaction

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Although all types of muscle cells use actin and myosin for contraction, only in skeletal and cardiac muscle these proteins are organized into sarcomeric units. These units are delimited by Z-discs, which are intricate webs of various proteins including α-actinin-2, F-actin and myotilin.At the focus of ourresearch is protein myotilin. Myotilin is a dimer,which consists of two immunoglobulin-like domains flanked by a unique serine-rich N-terminus and a short C-terminal tail and is known to interact with F- and G-actin, α-actinin-2, ZASP, FATZ and filamin C.In order to gain insight into its remarkable interaction capabilities we investigated its actin-binding properties from the structural point of view.

First, we determined binding affinities for interaction of different myotilin constructs with G-actin using microscale thermophoresis measurements, all showing relatively weak binding in the micromolar range. Binding to the F-actin was assessed with actin co-sedimentation assays, showing that Ig2 domain represents a focal point of interaction and Ig1 plays a supplementary role.

We additionally confirmed the binding of myotilin to G-actin using chemical cross-linking coupled with mass-spectrometry (XL-MS), which enabled us to identify amino acid residues likely involved in interaction. Furthermore, ^{15}N-HSQC titration NMR experiments were performed for the Ig1 domain to further map the binding site on myotilin to the β strands B, D and E. Similar assignment of Ig2 domain is in progress. Based on our gathered experimental data and the literature, we propose a model in which each Ig domain of myotilin dimer specifically binds to a single subunit of actin filament. Consequently, full-length myotilin, anchored on an actin filament with C-terminal Ig-like domains, is able to connect two adjacent α-actinins in the sarcomere with its N-terminus.

Liver phospholipid fatty acids composition in response to different types of diets in rats of both sexes

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Introduction

Gender related differences in fatty acids (FAs) composition of different lipid classes are well established. Arachidonic acid (AA, 20:4) and docosahexaenoic acid (DHA, 22:6) proportions are higher in liver and plasma phospholipids in female than male rats fed on standard chow diet. Female rats replete their DHA status more readily than males, probably due to a higher expression of liver desaturases which should be taken into account for specific nutritional recommendations. In liver phospholipids, females exhibited higher percentages of DHA, eicosapentaenoic acid (EPA, 20:5), and overall n-3, while the percentage of docosapentaenoic acid (DPA, 22:5 n-3) was lower, together with higher hepatic Δ-6 desaturase mRNA and protein content, which suggests that females have a higher capacity to synthesize DHA from shorter chain n-3 PUFA than males. This expression difference seems to be limited to the liver, as no differences in enzymes or transcription factors were observed in the heart or brain. The aim of the present study was to examine the effects of different diets, fish-based and milk-based *vs.* standard diet, on phospholipids fatty acids composition in rats liver of both sexes.

Materials and methods

Adult Wistar rats of both sexes (n = 8; 250–300 g) were used in this study. Animals were fed with different type of diets (standard, fish-based and milk-based). FAs methyl esters derivatives formed from isolated liver phospholipids fraction were separated by Gas Chromatography using Shimadzu GC 2014. Statistical significance of FAs levels was tested with two-way ANOVA using the sex of animals and treatment (type of diet) as factors on logarithmic or trigonometric transformed data. The trends were considered as significant if $p < 0.05$. The values were post hoc compared by Turkey's HSD test.

Results

In females, fish and milk based diet elevated eicosatrienoic acid (ETA, 20:3) ($p < 0.001$) level comparing to standard diet. Fish based diet lowered the amount of AA ($p < 0.001$) and increased levels of EPA ($p < 0.001$), DPA ($p < 0.001$) and DHA ($p < 0.001$) in relation

to standard diet. Milk based diet elevated levels of AA ($p = 0.001$) and docosateraenoic acid (DTA, 22:4) ($p = 0.001$).

In males, milk based diet decreased EPA ($p < 0.01$) and DHA ($p < 0.001$) and increased DTA ($p<0.001$) levels compared to controls. On the other hand, fish based diet elevated only DHA levels ($p < 0.001$) compared to standard diet ($p<0.001$).

Milk based diet decreased n-3 ($p < 0.001$), and increased n-6 ($p < 0.001$) and n-6/n-3 ($p < 0.001$) ratio in males comparing to the other type of diets. On the other hand, in females, fish based diet increased n-3 ($p < 0.001$), decreased n-6 ($p < 0.001$) and n-6/n-3 ratio ($p < 0.001$) comparing to standard and milk based diets (ANOVA diet effect, $p < 0.001$, S x D effect, $p < 0.001$, Tukey's post hoc).

Conclusions

In conclusion, gender related variations in fatty acid composition of rat liver phospholipids were observed, and our results have shown that those initial differences can be significantly modulated by the type of diet. Furthermore, the modulatory effects of milk- and fish-based diets on liver phospholipids FAs profiles appeared to be sex-specific.

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Gall formation by *Rhinusa pilosa* alters the carbohydrate composition in *Linaria vulgaris* stem

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Introduction

Gall is defined as atypical plant growth induced by host-specific organisms, and represents larval chamber which provides food and protection during insect development. Yellow toadflax, *Linaria vulgaris* (*Scrophulariaceae*) is the host plant of a highly specific stem-galling weevil *Rhinusa pilosa* (Gyllenhal, 1838).

Interactions between host plants and gall-inducing insects are remarkable examples of structural, physiological, and chemical manipulations of plant cells resulting in abnormal plant outgrowths – galls [1]. *Rhinusa pilosa* is a stem-galling weevil associated with yellow toadflax, *Linaria vulgaris* [2,3]. In this interaction, the insect provides the stimulus and the plant initiates the growth response. In our previous study, we used the highly specific interaction of *R. pilosa* and *L. vulgaris* to reveal the mechanisms of gall initiation [4].

Gall-inducing insects are mostly inert during the feeding stage [5], thus they are completely dependent on the strong nutrient sink formed in the host plant tissue. Recent studies have shown that leaf gall tissue has altered photosynthetic activity compared to uninfected one, accompanied by increased content of soluble sugars and changes in starch content [6,7]. However, studies on stem galls are scarce, as well as comparative analysis of soluble sugars composition during gall development [8].

The aim of this study was to determine changes in carbohydrates composition in *L. vulgaris* induced by insect feeding, gall initiation (less than 24 h, G1) and gall development (seven days, G2).

Soluble sugar analysis

For soluble sugar analysis, frozen tissues were homogenized in liquid nitrogen and extracted as described in Vidović et al. [9]. Chromatographic analysis of soluble sugar were performed using DIONEX ICS 3000 DP liquid chromatography system (Dionex, Sunnyvale, CA, USA) equipped with a quaternary gradient pump (Dionex). The

carbohydrates were separated on a CarboPac®PA100 pellicular anion-exchange column (4-250 mm) (Dionex). The main carbohydrates detected were monosacharides: arabinose, fructose, galactose, glucose, mannose, rhamnose, and xylose; disaccharides: maltose, sucrose, trehalose and turanose; trisaccharide raffinose, and sugar alcohols: galactitol, mannitol and sorbitol.

The content of carbohydrates between control stems at the beginning and at the end of the experiment was similar. In initiating gall, the most striking change in soluble carbohydrates composition was about 14-fold increased accumulation of trehalose, accompanied by significantly increased contents of glucose, sucrose, turanose, raffinose, and xylose ($p < 0.05$, Mann –Whitney test) compared to control plants (Figure 1). In the same time, concentrations of galactitol and sorbitol decreased compared to control stems. Seven days later, glucose content increased significantly compared to both controls and G1 (Figure 1).

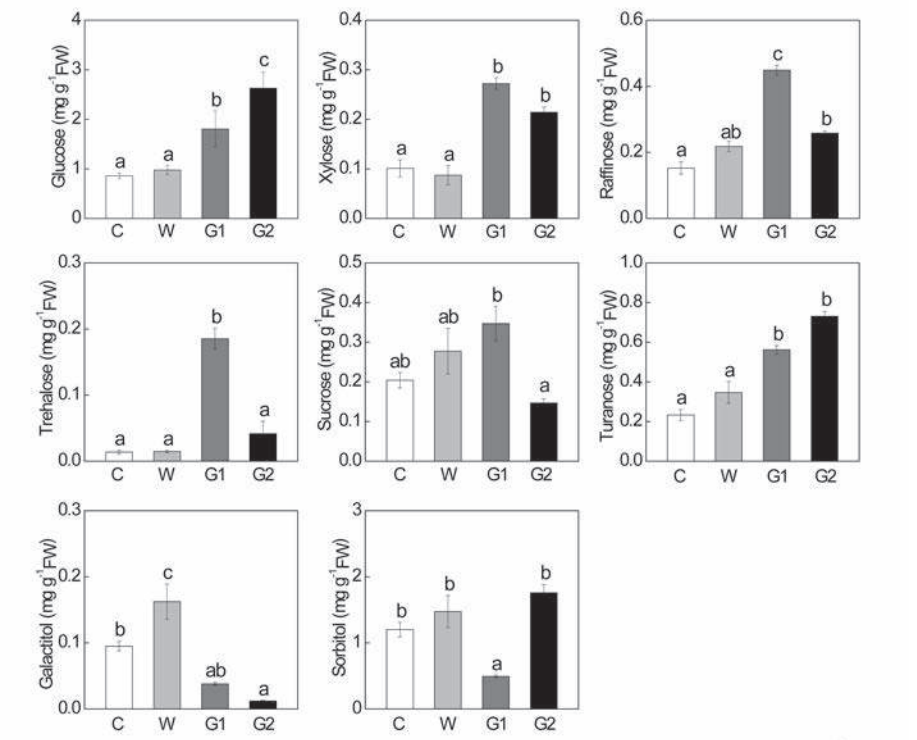

Figure 1. Content of soluble carbohydrates in *L. vulgaris* control stems (C), wounded stems after insect feeding (W), and in initiating gall (G1, less than 24h) and growing gall (G2, 7 days after oviposition).Values represent the means ± SE ($n \geq 4$). Different letters denote statistically significant differences between the treatments according to Tukey's post-hoc test.

Both sucrose and trehalose are important signalling metabolites which regulate specific responses to environmental signals such as diurnal changes and biotic and abiotic stress throughout the plant [4,8].

Content of starch and total water-insoluble carbohydrates

Extraction and determination of total water-insoluble carbohydrates and starch was performed according to modified protocols by Hansen and Møller [10] and Sadasivam and Manickam [11].

Starch content significantly decreased in wounded stems ($p < 0.001$, Mann-Whitney test) compared to controls, while a slight decrease was observed in G1 (Figure 2). On the other hand, the content of TIC increased 1.6-fold in wounded stems and G1 compared to control. Seven days after oviposition, accumulation of TIC continued to increase, reaching 2.1-fold higher value compared to control stems (Figure 2).

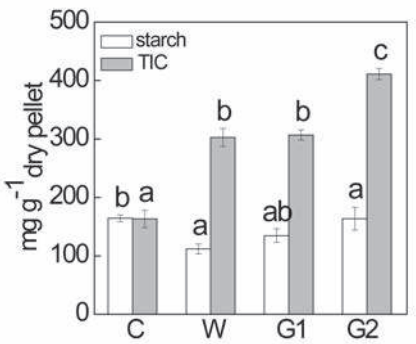

Figure 2. Content of starch and total insoluble carbohydrates (TIC) in *L. vulgaris* control stems (C), wounded *L. vulgaris* stems after insect feeding (W), and in initiating gall (G1, less than 24h) and growing gall (G2, 7 days after oviposition).Values represent the means ± SE ($n \geq 4$). Different letters denote statistically significant differences between the treatments according to Tukey's *post-hoc* test.

The experimental system presented in this work allows comparisons of carbohydrate profiles in time course, during gall initiation and growth, as well as distinguishing the effects of herbivory. The results obtained in this study indicate that *R. pilosa* can manipulate plant resource allocation pattern from the moment of oviposition to gall development. Increased accumulation of soluble carbohydrates and carbohydrates in the cell wall support the hypothesis that gall tissue acts as a strong sink of photoassimilates.

Acknowledgments

This research was supported by III43001 and III43010 project funded by the Ministry of Education, Science and Technological Development of the Republic of Serbia.

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Rational design of raw starch degradingα-amylasefrom *Bacillus licheniformis* 9945a for possible surface binding sites identification

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Raw starch degrading enzymes often possess extra substrate binding regions that enhance their activity to starch granule *via* physical adsorption. These can be found either on separate domains termed starch binding domains (SBDs) or in the form of surface binding sites (SBSs) situated on the surface of enzymes. Conservation of SBS is not known nor expected amongst amylase families. However, within same subfamily it can be expected to have occurrence of same or similar residues being involved in starch binding. Confirmed adsorption ofα-amylase from *Bacillus licheniformis* ATCC 9945a (*Bli*Amy), a potent enzyme for raw starch hydrolysison raw starch granules is indicativefor presence of SBS as enzyme is lacking SBD. Suspected sites responsible in *Bli*Amy were identified by homology modeling and *in silico* analysis. Site-directed mutagenesis of target amino acid residues was performed. Wild typeenzymeand mutantswere produced using an optimized fed-batch approach in a defined media with significant overexpression of 1.2 g L^{-1}. A mixed mode Nuvia™cPrime™ resin was used for downstream processing with yields of 96% directly from the fermentation broth. Role ofenzyme domain C in raw starch adsorption has been elucidatedby deletion analysis. The Langmuir model was applied for adsorption kinetics study of mutants to starch granules, while kinetics of hydrolysis was followed by Michaelis-Menten equation.

Acknowledgement

This work was supported by the Serbian Ministry of Education, Science and Technological development, project grant number 172048 and International Centre for Genetic Engineering and Biotechnology (ICGEB) research project grant number CRP/YUG11-02.

Eryngium serbicum as new source of cytotoxic and antibacterial agents

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The genus *Eryngium L*. is most complex and largest genus in *Apiaceae* family. *Eryngium serbicum* is endemic perennial herb, localized in the central part of the Balkan Peninsula. The species was designated as 'Vulnerable' according to IUCN categorization of the vulnerability. Species of this genus are traditionally used in many countries as cultivated, edible or medicinal plants [1]. In this study, we evaluated cytotoxic and antibacterial activity of *E. serbicum* methanol extracts, derived from aerial (flower, leaf and stem) and root plant organs.

Determination of the cytotoxic effects of methanol extracts of flower, leave, stem and roots obtained from *E. serbicum* was observed on colon cancer adenocarcinoma cell line HCT-116 and SW-480, human breast cancer cell line MDA-MB-231 and healthy human lung fibroblast MRC-5 by MTT cell viability assay [2]. The cytotoxic effects of extracts were expressed by IC_{50} (inhibitory dose which inhibit 50% growth cells). Obtained results of investigation (Table 1) indicated that flower, leaf and stem methanol extracts have the significant cytotoxic effects only on tumor cells, while methanol extract of roots showed cytotoxicity on tumor and healthy cells. From these results we can conclude that flower, leave and stem methanol extracts can be considered as suitable candidates for further studies as effective anticancer drug components.

Antibacterial activity determination was based on a microdilution method [3]. Antibiotic streptomycin was used as a positive control. Extracts were tested against seven gram positive (*Bacillus mycoides*, *Bacillus subtilis*, *Enterococcus faecalis*, *Micrococcus luteus*, *Micrococcus lysodeikticus*, *Staphylococcus aureus* and *Staphylococcus epidermidis*) and three gram negative bacteria (*Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*), and results obtained are presented as minimal inhibitory concentration (MIC_{50} values). In general, all tested extracts showed better antibacterial activity on gram positive bacteria. For extract obtained from roots and flowers MIC_{50} values are in range of 15.6–125 μg/mL, while for leaves extracts MIC_{50} values are in range of 15.6–250 μg/mL.

Extracts obtained from stems showed MIC_{50} values in range of 62.5–250 μg/mL. Gram negative bacteria *E. coli* was found to be the most resistant on all tested extracts. Flowers, leaves and stems methanol extracts had strong potency against gram positive *M. lysodeikticus* and *M. luteus*, while roots extracts showed the highest antibacterial activitie on gram positive *B. mycoides* and Gram negative *K. pneumoniae*.

Table 1. *In vitro* growth inhibitory activity (IC_{50} μg/mL) of *E. serbicum* methanol extracts of flowers, leaves, stems and roots on HCT-116, SW-480, MDA MB-231 and MRC-5 cell lines after 24 and 72 h of exposure. Values are presented as mean ± SE from 3 experiment.

	IC_{50} (μg/mL)	
Cell lines and extracts	24 h	72 h
HCT-116		
Flowers	375.39 ± 0.46	17.96 ± 0.43
Leaves	209.56 ± 0.32	65.44 ±0.50
Stems	>500	65.97 ±0.46
Roots	89.80 ± 0.82	83.99 ± 0.81
SW-480		
Flowers	54.25 ± 0.46	23.03 ± 0.47
Leaves	> 500	12.96 ± 0.39
Stems	49.24 ± 0.36	20.25 ± 0.46
Roots	5.78 ± 0.53	24.76 ± 0.77
MDA-MB-231		
Flowers	224.03 ± 0.62	54.23 ± 0.63
Leaves	121.24 ± 0.33	15.93 ± 0.21
Stems	246.97 ± 0.67	30.81 ± 0.43
Roots	38.73 ± 0.53	15.14 ± 0.56
MRC-5		
Flowers	>500	>500
Leaves	>500	>500
Stems	>500	>500
Roots	156.50 ± 0.61	227.20 ± 0.31

Overall, this research provided a strong indication of the potential use of *E. serbicum* different plant organs as cytotoxic and antibacterial agents.

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Notes

Notes

ALFA GENETICS

Alfa Genetics d.o.o. je autorizovani distributer, uvoznik i ovlašćeni serviser renomiranih evropskih proizvođača medicinske i laboratorijske opreme i potrošnog materijala. Vrhunski kvalitet proizvoda prate kvalitet usluga koje pružamo u pogledu stručne pomoći prilikom odabira odgovarajuće opreme, logistike, montaže i održavanja u garantnom i vangarantnom roku.

Asortiman delatnosti obuhvata širok spektar medicinskih aparata i opreme, potrošnog materijala, hemikalija i reagenasa, dijagnostike i ostalih relevantnih oblasti. Prodajni program čine proizvodi svetskih renomiranih proizvođača:

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ELTA 90 Medical Science je najmlađi član ELTA 90 grupacije, regionalnog lidera u distribuciji medicinskih sredstava, laboratorijske opreme i potrošnog materijala. Sa više od 15 godina iskustva u regionu, ELTA 90 je gradila svoju reputaciju uspešne kompanije, kojoj su partneri na prvom mestu.

Veliki broj renomiranih svetskih kompaniija koje zastupamo svedoči o našem poslovanju koje se bazira na stalnoj edukaciji, unapređenju kvaliteta i brzine usluge, inovacijama, društvenoj odgovornosti i građenju poverenja i partnerskih odnosa sa našim klijentima. Profesionalne potrebe i razvoj naših klijenata su bile i ostale naš prioritet.

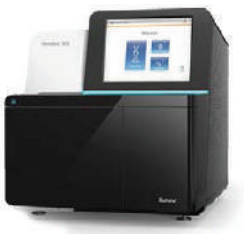
Kompanija **Illumina** je osnovana 1998. godine u San Dijegu, Kalifornija. Trenutno ima preko 6000 zaposlenih i predstavlja globalnog lidera u genetici, polju koje povezuje biologiju i tehnologiju. Nakon Projekta humanog genoma, kada je prvi put odgonetnuta kompletna primarna sekvenca molekula DNK i kada je identifikovano i mapirano preko 20 000 gena, istraživanja su prešla na još veću skalu. Ovaj naučni proboj doprineo je da molekularna genetika i studije na čitavom humanom genomu postanu fundamentalan deo medicine i brige o zdravlju ljudi. Polje kliničke molekularne dijagnostike se u poslednjoj deceniji značajno proširilo, pre svega zahvaljujući razvoju nauke i tehnologije. Tehnologija sekvenciranja nove generacije (eng. New Generation Sequencing, NGS) predstavlja novu i revolucionarnu metodu kojom je moguće analizirati hiljade gena u samo jednom koraku. Kompanija Illumina je pionir u razvoju ove metodologije, a nedavno je proslavlljajući svoj 18. rođendan, potvrdila svoj položaj lidera u ovoj oblasti. Osim dijagnostičkih analiza, koje su postale rutinske na Illumininim aparatima poput MiniSeq-a i MiSeq-a, omogućavamo istraživačima da idu još dalje. Vođeni željom za novim saznanjima, kako bi pronašli odgovore na sva pitanja koja intrigiraju naučnu javnost i javnost uopšte, svakoga dana se razvijamo. Pristup Ilumina grupi ne samo da omogućava razmenu iskustva i znanja, već pruža naučnicima mogućnost da osete pokretačku enegriju koju nosi rad na sekvenatorima nove generacije. Omogućava im da budu prvi i da pomeraju granice. Pozivamo i Vas da napravite razliku, pridružite se Illumininoj zajednici.

Uz Illuminu, budućnost je sada.

Ono što izdvaja Illuminu od drugih proizvođača jeste posebna tehnika sekvenciranja sintezom – ***SBS (Sequencing by synthesis)*** čija je osnovna prednost velika osetljivost i smanjenje mogućnosti greške, kao i ***"Pair-end sequencing"*** koji omogućava dodatnu proveru sekvenciranja.

MiniSeq - nova super zvezda Illumine. Sva snaga u malom, pristupačnom i jednostavnom za upotrebu instrumentu najnovije generacije.

MiSeq –fokus na snazi i neograničenim mogućnostima. Ciljano sekvenciranje gena, metagenomika, sekvenciranje malih genoma, ciljana ekspresija gena, sekvenciranje amplikona, HLA tipizacija uz Output do 15Gb!



NextSeq 500 - najjačidesktop sekvenator, objedinjuje veliku snagu, fleksibilnost i jednostavnost u radu, kako bi Vam osiguraošto jednostavniji pristup u izučavanju kompletnog genoma, epigenoma i transkriptoma.

HiSeq sistemi - neprikosnoveni sistemi prepoznatljive snage, brzine i efikasnosti koji su postali prva opcija za sve veće genetičke centre širom sveta.

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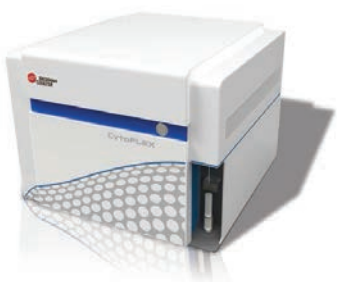
Kompanija **Beckman Coulter** je osnovana pre 80 godina, a do danas ima predstavništvo u preko 130 zemalja sveta i broji preko 11 800 zaposlenih. Beckman Coulter je predano posvećen unapređivanju i optimizovanju aparata i reagenasa u laboratorijama. Tokom godina razvijeno je veliko poverenje između klijenata i ove kompanije, tako da se za ime Beckman Coulter pre svega vezuje reč "kvalitet". Glavni fokus zaposlenih u Beckman Coulter-u je inovacija, pouzdanost i efikasnost, zbog čega se njihov uspeh na tržištu laboratorijske opreme veoma opravdano povećava iz godine u godinu.

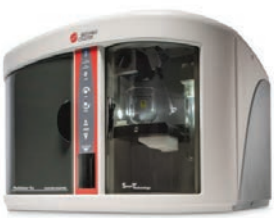
Beckman Coulter Life Sciences

Beckman Coulter Life Sciences pruža inovativna i pouzdana rešenja za laboratorije sa najrazličitijim potrebama širom sveta. Njihovu opremu ćete videti na univerzitetima, u vladinom sektoru, u biotehnološkim i farmaceutskim kompanijama, bolnicama i komercijalnim laboratorijama. Svojim najsavremenijim pristupom Beckman Coulter Life Sciences preispituje konvencionalne metode kako bi razvio najbolje moguće proizvode koji su globalno prepoznati kao izuzetno pouzdani, a samim tim su i veoma rado i sa ponosom korišćeni u laboratorijama.



Iz Beckman Coulter Life Sciences portfolia izdvaja se program za protočnu citometriju, koji je posebno prilagođen jedinstvenim potrebama istraživača (**CytoFLEX aparat**) kao i širok dijapazon centrifuga (Allegra, Avanti i Optima serije).

Beckman Coulter Industry



Svaki korak u industrijskoj proizvodnji iziskuje precizno merenje, te samim tim istraživanje, razvoj i brza proizvodnja direktno zavise od opreme koja se koristi u datoj kompaniji. Beckman Coulter Industry pruža potpuno integrisane, lake za upotrebu, automatizovane sisteme sa najrazličitijom primenom u kontroli kvaliteta – merenje čestica, praćenje njihove distribucije i zapremine koje zauzimaju kao i kompletna analiza na ćelijskom nivou. Svi Beckman Coulter sistemi mogu da se konfigurišu tako da izađu u susret specifičnim potrebama svih zaposlenih u industriji i pružaju efikasan proces automatizacije za najrazličitije korake u trgovini.

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