Serbian Biochemical Society
Fifth Conference

Faculty of Chemistry, University of Belgrade,
13.11.2015. Belgrade, Serbia.

"Integrated research in life science"

PROGRAMME

10.00-10.10	Welcome message and information about SBS and FEBS upcoming events Prof. M. B. Spasić (President of the Serbian Biochemical Society)
10.10-10.30	Milan R. Nikolić Department of Biochemistry, Faculty of Chemistry, University of Belgrade, Belgrade, Serbia **Hemoglobin: new faces of the old lady**
10.30-10.50	Jelena Kotur-Stevuljević Department for Medical Biochemistry, Faculty of Pharmacy, University of Belgrade **PON1 enzymes, analytical possibilities: ten years of experience**
10.50-11.00	Coffee break
11.00-11.20	Miroslav Nikolić Plant Nutrition Research Group Institute for Multidisciplinary Research, University of Belgrade **Root-induced changes at the soil-plant interface: from biochemistry to agriculture**
11.20-11.40	Željko Popović Department of Biology and Ecology, Faculty of Sciences, University of Novi Sad **Molecular regulation of insect diapause**
11.40-12.00	Tijana Stanković Department of Neurobiology, Institute for Biological Research "Siniša Stanković", University of Belgrade **Evaluation of CXCR4 receptor and FAK kinase as possible targets for non-small cell lung carcinoma treatment**
12.00-13.00	Poster Session
13.00-13.30	Coctail

13.30-13.50	Polina D. Blagojević Department of Chemistry, Faculty of Science and Mathematics, University of Niš **Discovery of anxiolytic 2-ferrocenyl-1,3-thiazolidin-4-ones exerting GABAA receptor interaction via the benzodiazepine-binding site**
13.50-14.10	Jelena Bogdanović Pristov Department of Life Sciences, Institute for Multidisciplinary Research, University of Belgrade **Cell wall: active component of redox metabolism in plants**
14.10-14.30	Ivana Nikolić Department of Biochemistry, Faculty of Medical Sciences, University of Kragujevac **Induction of apoptosis in human endometrial stromal cell line with different substances**
14.30-15.00	Discussion and concluding remarks

Poster Session

Jelena Janković
Institute for the Application of Nuclear Energy – INEP, Department for Endocrinology and Radioimmunology, University of Belgrade
Malignant risk stratification of thyroid fine needle aspiration specimens using serum, genetic and protein markers

Bojan Jevtić
Department of Immunology, Institute for Biological Research "Siniša Stanković", University of Belgrade, Serbia
Cucurbitacin E inhibits effector cytokine production in encephalitogenic cells

Gordana Kovačević
Faculty of Chemistry, University of Belgrade
Semi rational design of glucose oxidase from *Aspergillus niger* for oxidative stability

Ana Mijušković
Department of Physiology, Institute for Biological Research, University of Belgrade
Calcium-activated chloride channels and Na_2S-induced relaxation of non-pregnant rat uteri in estrus

Goran Miljuš
Institute for the Application of Nuclear Energy (INEP), University of Belgrade
Isolation, characterisation and the role of complexes formed between transferrin and insulin-like growth factor-binding protein 3

Jelena Mihailović
Faculty of chemistry, University of Belgrade
Lysine acetylation of major Chlamydia trachomatis serovar B antigens

Jasna Nikolić
Department of Biochemistry, Faculty of Chemistry, University of Belgrade
Development of high resolution 2D map of *Musa acuminata* proteins using combinatorial peptide ligand libraries for the detection of novel allergens

Ivan D. Pavićević
Department of Biochemistry, Faculty of Chemistry, University of Belgrade
Fatty acids change the reactivity of the human serum albumin Cys34 thiol group

Milica Radibratović
ICTM, University of Belgrade
Binding and Molecular Dynamics Studies of Phycocyanobilin with Human Serum Albumin

Brankica G. Rašković
Department of Biochemistry, Faculty of Chemistry - University of Belgrade
Cold stability of proteases

Dragana Robajac
Institute for the application of nuclear energy – INEP, University of Belgrade
N-glycome of membrane proteins and receptors for insulin and insulin-like growth factors, isolated from human placenta from different (patho)physiological conditions

Mariana Seke
Institute of Nuclear Sciences ‘’Vinča’’, University of Belgrade
Characterization of fullerenol/doxorubicin nano composite, its cytotoxicity in malignant cell lines along with cardio- and hepato- protection in rats

Marinela Šokarda Slavić
Centre of Chemistry, Institute of Chemistry, Technology and Metallurgy, University of Belgrade
Mixed-mode resins: taking shortcut in downstream procesof raw-starch digesting α-amylases

Miloš Šunderić
Institute for the application of nuclear energy – INEP, University of Belgrade
Molecular forms of IGF binding protein 2 and their presence in various pathophysiological states

Milica Vujičić
Department of Immunology, Institute for Biological Research “Sinisa Stankovic”, University of Belgrade
Old herbs for the new therapy of type 1 diabetes

Foreword

Dear Colleagues,

It is my great pleasure to wish you warm welcome to the 5th Conference Conference of the Serbian Biochemical Society entitled "Integrated research in life science".

Official languages for 5th Conference of the Serbian Biochemical Society will be Serbian and English. We have invited eight lecturers from Serbia to present their achievements in their respective fields of work and their presentations will be published in the Proceedings. For the first time we invited students of PhD studies from Belgrade University to present their work in form of Posters and their presentations will be published in our Proceedings as Abstracts or extended Abstracts.

Please find enclosed information on 41th FEBS Congress to be held on 03 – 08 September, 2016 in Kusadasi, Aydin, Turkey.

I would like to express my gratitude to the members of the governing board of the Serbian Biochemical Society who suggested lecturers and to all of those who accepted the invitation.

Editor of the Proceedings
Prof. Mihajlo B.Spasić
President of the
Serbian Biochemical Society

Proceedings

Hemoglobin: new faces of the old lady

Milan R. Nikolić [1]

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Because of its red color, the red blood cell protein hemoglobin has been of interest since antiquity. Many 'firsts' are associated with hemoglobin, e.g. first protein to be crystalized, first protein to have its mass accurately measured, or first protein to be studied by ultracentrifugation. The X-ray crystallography of hemoglobin and myoglobin, its smaller cousin, provided the first three-dimensional images of proteins and led to the concept of protein-fold conservation during divergent evolution. The term "molecular disease" was introduced after the discovery that the structure of cycle cell hemoglobin differed from that of normal hemoglobin. Hemoglobin has been taken as a prototype for studying structure-function relationship in proteins, as a paradigm for cooperative systems in biochemistry and allosteric modulation of ligand binding. In the last 20 years, many new aspects of hemoglobin biochemistry have been discovered or clarified. The aim of this text is to give a brief and concise overview of several parallel physiological function of this fascinating molecule apart from its basic function of oxygen transport. The more significant of these include: a much more detailed understanding of the structural factors that regulate oxygen transport function; hemoglobin as a source of physiological active metabolites; enzymatic activities of (different) hemoglobin forms; the discovery of new classis of hemoglobin in plants, and hemoglobin as a high capacity buffer system for cholesterol in the bloodstream.

Introduction

Large organisms have a high respiratory demand for oxygen. Hemoglobin (Hb), as the major protein in vertebrate erythrocytes and one of the vital biomolecule in nature, is involved in oxygen transport from the lungs to the tissues. Every milliliter of human blood has approximately 5 billion erythrocytes and each erythrocyte is packed with about 280 million molecules of hemoglobin. Because hemoglobin is found in high (millimolar) concentrations *in vivo*, it has been the object of great number of studies since the development of the life sciences. Several hemoglobins appear successively during ontogenic evolution and a different pattern of hemoglobin molecules is present in the various stages of the human life cycle; more than 200 variant and abnormal hemoglobins have been described so far. The name 'hemoglobin' emerged from their compact, globular structure and use of a heme prosthetic group to bind oxygen. Since the uptake and delivery of oxygen is such an essential process, the hemoglobin molecule has been carefully engineered to display a fine

tuning of its oxygen-carrying properties, characterized by the presence of both homo- and heterotropic interactions, in order to ensure an adequate delivery of oxygen in response to the physiological demands of the containing species.[1]

The textbook view to general hemoglobin structure and function[2-9]

The structure of hemoglobin has been extensively studied by X-ray crystallography. For this endeavor Dr Max Perutz was awarded the Nobel Prize in chemistry in 1962. In the vertebrates, the hemoglobin contained in the red blood cells is a tetramer that consists of two pairs of unlike polypeptide chains. One chain of each dimer is α or α-like chain while the other is non α (β, γ or δ) chain. α chains of all human hemoglobins are alike. The non-α chains include the beta chain of normal adult hemoglobin (HbA: $\alpha_2\beta_2$), the gamma chain of fetal hemoglobin (HbF: $\alpha_2\gamma_2$), and the delta chain of hemoglobin A2 ($\alpha_2\delta_2$). The α-subunits and β-subunits are made up of 141 and 146 amino acids forming 7 and 8 α-helices, respectively, with the helices (named from A–H) joined by non-helical segments. Each of the four subunits in hemoglobin contains one prosthetic heme group iron (protoporphyrin IX) that is bound in a deep pocket formed by the E and F helices. The heme ferrous atom is coordinated by the four porphyrin nitrogens at the equatorial plane, as well as an axial proximal histidine, His(F8), namely αHis87 and βHis92. The distal side of the heme is characterized by a pocket where molecular oxygen and other ligands bind to the heme iron. When it is five coordinated (no ligand at the distal position), the iron moves out of the porphyrin plane toward the proximal histidine, and binding of ligand at the distal side pulls the iron into the plane of the porphyrin.

The major physiological function of hemoglobin is to facilitate the movement of oxygen from the lungs to the tissues within the physiological range of oxygen partial pressures in arterial and venous blood. This role is regulated and/or made efficient by numerous endogenous heterotropic effectors. In solution, hemoglobin is in an allosteric equilibrium between two alternative functional states, the tense one (T) with low (the deoxy state), and the relaxed one (R) with high oxygen affinity (the oxy state). The two αβ dimers are arranged around a 2-fold axis of symmetry, resulting in a large central water cavity in the deoxy structure and a narrower cavity in the oxy (liganded) structure. Successive oxygen binding shifts the allosteric equilibrium toward the R higher-affinity states, to explain the sigmoid shape of the hemoglobin-oxygen dissociation curve. This can be linked to a molecular form of paradoxical breathing: unlike the lungs, the hemoglobin molecule contracts when oxygen enters and expands when oxygen leaves. The transition between two alternative hemoglobin quaternary structures consists of a rotation of one αβ-dimer relative to its partner, accompanied by shifts at the α1bβ and α2β1 contacts. The T structure is constrained by salt-bridges between its four subunits, formed by the C-terminal arginine residues of the α-subunits and the C-terminal histidine residues of the β-subunits. These bridges are absent in the R structure, which has an oxygen affinity similar to that of free α and β-subunits, while the oxygen affinity of the T structure is lower by the equivalent of about 3 to 3.5 kcal/mol heme depending on conditions.

The T and R quaternary structures were used to validate the two state Monod–Wyman–Changeux (MWC) model for the mechanism of allosteric proteins. Upon ligand binding, the T state switches to the R state without intermediate states. An alternative mechanism, known as the Koshland–Némethy–Filmer (KNF) model, assumes that, without ligand, hemoglobin exists in only one conformation and ligand binding induces a conformational change that are transmitted to other subunits. Perutz proposed a detailed stereochemical mechanism for the cooperative effects in hemoglobin, embodying aspects of both the MWC and KNF models. He postulated that each subunit of the tetramer goes through tertiary conformational change upon ligand binding, which is then transmitted to another subunit through direct communication between the α1 and β2 subunits, leading to sequential increase in the affinity for ligand at other heme sites and thereby shifting the equilibrium toward the R state. As a result the forth heme has a very high oxygen affinity and binds oxygen readily. The physiological significance of cooperativity and of the sigmoidal shape of the oxygen dissociation curve consists in the fact that the release of oxygen from fully oxygenated hemoglobin in tissue capillaries results in a decrease in the affinity for the remaining oxygen. This increase in the slope of the oxygen dissociation curve provides for the delivery of large amounts of oxygen but at the same time preserves an oxygen partial pressure gradient adequate to assure oxygen diffusion into the tissue.

Physiological regulators of the oxygen binding are hydrogen ions (Bohr effect), carbon dioxide and organic phosphates, intermediates in red cell glycolysis, particularly 2,3-diphosphoglycerate (2,3-DPG). Their increasing concentration tends to stabilize the deoxy conformation of the hemoglobin tetramer and decrease oxygen affinity. The effect of hydrogen ions is associated with the observation that deoxyhemoglobin is a weaker acid than oxyhemoglobin. Carbon dioxide decreases oxygen affinity both through its effects on pH and by binding to N-terminal amino acid residues of each subunit to form carbamino derivates. The effects of 2,3-DPG is mediated by its binding to a stereochemically complementary site in the central cavity of deoxyhemoglobin, forming additional salt bridges between the β chains to stabilize further the deoxy, low affinity conformation. An increase in 2,3-DPG also results in a decrease in intraerythrocytic pH which secondarily decreases the oxygen affinity by the Bohr effect. Gases other than oxygen, such as carbon monoxide and nitric oxide, also are able to combine with the ferrous atom of hemoglobin. Carbon monoxide attaches itself more firmly (210 times greater affinity), and oxygen cannot displace carbon monoxide from hemoglobin to any extent. The chemical stability of carboxyhemoglobin has made carbon monoxide a ligand of choice for studying the R state, and the majority of liganded hemoglobin structures in the PDB are in this form.

Enzymatic (redox) activities of hemoglobin and its consequences

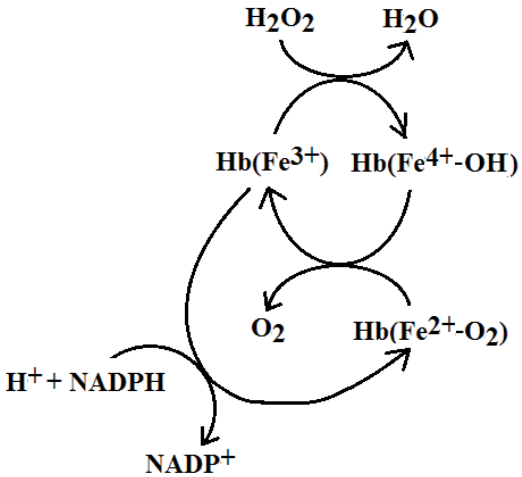
Hemoglobin has a high affinity for oxygen and a low affinity for carbon dioxide, organic phosphates, and hydrogen and chloride ions in the arterial circulation. In the venous circulation, these relative affinities are reversed. To highlight these remarkable properties,

Dr Jacques Monod conferred on hemoglobin the title of "honorary enzyme". Indeed, if heme is active site, oxygen is substrate, and hydrogen ion is inhibitor, then hemoglobin mimics the properties of an enzyme. Now, when we know that hemoglobin in the red blood cell continuously undergoes redox reactions, the "honorary enzyme" hemoglobin turns out to be a "real enzyme".[10]

Redox reactions associated with autoxidation[11-17]

Redox-active transition metals, with unpaired elections in their *d* orbitals, are able to generate or remove various reactive species. Heme proteins like hemoglobin contain redox-active metal iron that makes them susceptible to causing oxidative damage. Although the structure of the globin chain allows heme to bind oxygen with minimal oxidation of ferrous to ferric iron, slow spontaneous autoxidation to yield superoxide anion ($O_2^{-\bullet}$) is not entirely prevented. Autoxidation comes along with the removal of an electron from the heme iron and requires the nucleophilic displacement of the bound oxygen in partially oxygenated hemoglobin by the distal histidine. Produced nonfunctional methemoglobin, a form of oxidized (ferric) hemoglobin, is normally maintained as a very small proportion of total hemoglobin (<1%), primarily by the action of red cell methemoglobin reductase activity. If the globin structure is destabilized, methemoglobin converts to hemichrome (the main constituent of Heinz bodies), in which either the distal histidine or an external ligand occupies the sixth coordination position of the ferric heme**.** Fully oxygenated hemoglobin is particularly stable with negligible rate of autoxidation; it increases several orders of magnitude at low partial pressures of oxygen when hemoglobin is partially oxygenated.

Hemoglobin is highly reactive with hydrogen peroxide (H_2O_2) that is a two-electron oxidant, initiating a cascade of secondary oxidative reactions. Therefore, hydrogen peroxide formed after superoxide anion dismutation may react with ferrous and ferric hemoglobin to produce the highly reactive cytoxic ferrylhemoglobin. In the reaction with ferric hemoglobin, hydrogen peroxide will use one electron to oxidize ferric hemoglobin into ferrylhemoglobin, and the other electron to oxidize the protein, generating a globin tyrosyl-based radical. In the reaction of a second molecule of hydrogen peroxide with ferrylhemoglobin a superoxide radical is produced, which can attack the porphyrin before it escape from the heme pocket. This complex further react with hydrogen peroxide resulting in the degradation of the heme, releasing iron and forming several fluorescent degradation products. In the presence of free ferrous iron released from heme, superoxide anion and hydrogen peroxide may react ("Fenton" chemistry) to generate the highly reactive hydroxyl radical ($^{\bullet}OH$). In addition to damage of erythrocytes itself, red blood cell-derived reactive species mediate cellular injury in tissues. Disturbing the cellular redox state, they influence many signal transduction pathways, notably nuclear factor kappa B and hypoxia-inducible factor-1 alpha, and their target genes. The pseudo-peroxidase activity of hemoglobin could also be involved in cellular and organ injury, and generation of vasoactive compounds.



Sheme I. Peroxidase activity of hemoglobin: Continuous reduction of hydrogen peroxide to water is obtained when methemoglobin ($Hb(Fe^{3+})$) is oxidized to ferrylhemoglobin ($Hb(Fe^{4+}\text{-}OH)$); oxyhemoglobin ($Fe^{2+}\text{-}O_2$) is regenerated when methemoglobin reductase uses NADPH to reduce $Hb(Fe^{3+})$.

Ascorbate (effective in reducing the formation of ferrylhemoglobin and tyrosyl-based radicals) and glutathione (conjugated with hemoglobin protects heme against oxidative challenges) antioxidant system is essential in attenuation of the pro-oxidant potential of redox active hemoglobin. Human hemoglobin antioxidant potential is less well understood. The reactivity of the only one reactive cystein (β93Cys) residue toward thiol-reactive agents is allosterically controlled by oxygen-dependent changes in hemoglobin conformation: R state is more reactive toward nitrosating and alkylating agents and to mercurials than T-state. This amino acid residue also affects electron transfer reactions and limit ferrous heme-derived superoxide production and reactivity.

Aging of the erythrocytes prompts the weakening of their antioxidant systems. This results in excessive hemoglobin oxidation and formation of reactive oxygen and heme species that oxidize the membrane lipids, decreasing red blood cells deformability and causing hemolysis and release of free hemoglobin into plasma. Plasma does not have as sophisticated and robust complementary network of antioxidant defenses as erythrocytes. As an illustration, three independent enzymatic systems (catalase, selenium-dependent glutathione peroxidase, and peroxiredoxin-2) reduce hydrogen peroxide to water. Further, in the presence of free transition-metal catalysts, particularly heme iron, or a state of pro-oxidant insult, some of strong reducing agents and potent antioxidants in plasma may also act as pro-oxidants. To avoid toxicity, free hemoglobin must be immediately bound to protein haptoglobin and

enter the monocyte/macrophage scavenger receptor CD163, to be catabolized by heme oxygenase-1 into antioxidant biliverdin/bilirubin and vasodilatory carbon monoxide. Free heme molecules, released from hemoglobin, binds with hemopexin to be detoxified by heme oxygenase-1 coupled with the low density lipoprotein receptor-related CD91 protein. Free ferric iron, the breakdown product of heme, binds to transferrin to encounter its receptor to be intracellularly stored, complexed with ferritin. Free ferrous iron binds to apoferritin and is stored in the ferric state. During massive hemolysis, due to infections, trauma, sickle cell anemia, and thalassemia or following transfusion of redox-active hemoglobin-based blood substitutes, the free hemoglobin/heme concentration exceeds the capacity of the plasma detoxifying system. In such conditions, their oxidative and nonenzymatic transformations can have serious consequences, e.g. systemic inflammation, endothelial dysfunction, and thrombosis.

Redox reactions involving nitrite[18-21]

Nitric oxide ($^{\bullet}NO$), a relatively stable free radical, is an important signaling molecule that plays a critical role in the regulation of smooth muscle relaxation, endothelial adhesion molecule expression, and platelet activation and aggregation. Nitric oxide is unstable in the presence of oxygen, forming dinitrogen trioxide and nitrite in plasma and limits the amount of nitric oxide that enters the red blood cell. The presence of low levels of nitric oxide on the β93 cysteine residue of hemoglobin (S-nitrosohemoglobin) has been the basis for a hypothesis that the erythrocyte may actually help nitric oxide transport to the vasculature. Now it is clear that the nitrite anion (NO_2^-; pK_a 3.2) is a major storage pool of bioactive nitric oxide under hypoxic conditions in mammals. In this sequence of event, the heme proteins hemoglobin and myoglobin reduce nitrite to nitric oxide to supplement its production from the classic L-arginine-nitric oxide synthase nitric oxide pathway that requires oxygen.

The production of labile bioactive nitric oxide in the red blood cell has been attributed to a redox reaction between nitrite produced in the plasma and hemoglobin. This reaction requires deoxygenated hemoglobin and thus a low partial pressure of oxygen. The initial step in the reduction of nitrite bound to ferrous iron atom (via uncommon *O*-nitrito binding mode) involves the release of a hydroxide ion from the protonated nitrite, resulting in a nitrosonium cation (NO^+) bound to the ferrous heme. The electron transfer from the ferrous iron to the nitrosonium ion produces ferric iron (methemoglobin) and nitric oxide. The methemoglobin reductase can re-reduce the methemoglobin generated by formally nitrite reductase hemoglobin activity back to the ferrous form, preventing its accumulation. The nitrosonium ion is a much stronger oxidizing agent than oxygen and, while the redox reaction with oxygen is very inefficient, a major portion of the nitrosonium ion bound to hemoglobin is reduced to NO. Since the affinity of nitric oxide to heme ferric iron is much lower than to ferrous heme iron, this redox reaction results in the formation of a labile form of nitric oxide. In this labile form, nitric oxide can escape of the erythrocyte providing a source for nitric oxide regulation of blood flow in the microcirculation. Reaction between

nitrite and oxyhemoglobin results in the formation of methemoglobin and nitrate, without the direct transfer of an electron from the heme iron to the nitrite. As well as for hemoglobin autoxidation, the distal histidine is essential for nitrite reduction mediated by hemoglobin. Increased flexibility in the heme pocket of unliganded or partially liganded hemoglobin facilitates the required interactions of the distal histidine.

$$Hb(Fe^{2+}\text{-}O_2) + {}^{\cdot}NO \longrightarrow Hb(Fe^{3+}) + NO_3^-$$
$$Hb(Fe^{2+}) + {}^{\cdot}NO \longrightarrow Hb(Fe^{2+}\text{-}NO)$$
$$Hb(Fe^{3+}) + {}^{\cdot}NO \rightleftharpoons Hb(Fe^{3+}\text{-}NO)$$
$$Hb(Fe^{2+}) + NO_2^- + H^+ \longrightarrow Hb(Fe^{3+}) + {}^{\cdot}NO + OH^-$$

Sheme II. Redox reactions of hemoglobin involving bioactive nitric oxide

Hemoglobin nitrite reduction is an important reaction under ischemic conditions. At intermediate oxygen pressures where both superoxide and nitric oxide are produced, the potential toxicity of the nitric oxide formed is boosted by the rapid reaction between nitric oxide and superoxide anion producing the highly reactive peroxynitrite ($ONOO^-$). Accordingly, hemoglobin redox reactions under hypoxic conditions produce a number of reactive species (superoxide anion radical, ferrylhemoglobin, free iron, heme degradation products, nitric oxide and peroxynitrite) increasing oxidative stress.

Not so "sweet" hemoglobin modifications[22-25]

Among several glycosylated major hemoglobin (HbA) species in minor amounts in normal human blood, HbA1c is of particular importance. Glycation of N-terminal valine residues of β chains occurs in a two-step Maillard reaction. This non-enzymatic process involves the initial formation of a labile Schiff base, which undergoes a subsequent Amadori rearrangement, forming an Amadori product, which in this case is HbA1c. Extent of glycosylation depends on the plasma concentration of a particular hexose. Hemoglobin A1c has been adopted by the World Health Organization into its recommended criteria for diabetes diagnosis. Three amino acid residue targets for fructation of HbA are αLys7, αLys127 and βLys66.

The Amadori product can undergo oxidative cleavage, resulting in the formation of advanced glycation end products (AGEs). The presence and accumulation of AGEs in many different cell types adversely affect extracellular and intracellular structure and function. AGEs block nitric oxide activity in the endothelium and provoke the production of reactive oxygen species. Activation of AGEs receptor causes upregulation of the transcription factors that play critical role in inflammation. In human erythrocytes, prolonged glycation generates structural modification and functional changes of hemoglobin. Allosteric structure of glycated hemoglobin is fixed in T state and exhibit moderately high oxygen affinity compared to non-glycosylated hemoglobin (HbA0). Hydrogen peroxide-induced iron

release is more from HbA1c than that from HbA0. Autoxidation and formation of carbonyl content, an index of oxidative stress, is higher by HbA1c. Similar findings were obtained with hemoglobin covalently modified by fructose molecule(s), suggesting a mechanism of increased formation of free radicals and oxidative stress in diabetes mellitus.

Hemoglobin and cholesterol: "There's a secret connection"[26-29]

In human, cholesterol (Ch) serves three mail functions: most important, it is a main component of cell membranes and structures, it helps the liver to produce bile acids, essential compounds to the digestion of fats, and it is used to manufacture all steroid hormones, including sex hormones. Regardless, dietary cholesterol is often portrayed by media as a poison. One thing remains certain: a high level of cholesterol in the circulation is the major factor, along with high blood pressure and smoking, contributing to coronary heart disease, the leading cause of death in the world. The problem with cholesterol begins when the body has too much of it, or has deposits in the wrong places, e.g. inside the walls of the heart's coronary arteries. There it contributes to the formation of atherosclerotic plaques. Fundamentally, atherosclerosis is a lipoprotein-driven disease, due to the fact that low-density lipoprotein, the main cholesterol-carrying particles, is exquisitely susceptible to oxidative damage. Low-density lipoprotein is therefore often referred as the "bad cholesterol" because it plays a key role in depositing cholesterol within arteries. High-density lipoprotein is termed "good cholesterol" because it helps remove cholesterol from artery walls and transport cholesterol to the liver for excretion.

Our serendipitous observation, that a portion of cholesterol (associated with phospholipid) could be firmly bound to hemoglobin in normal human erythrocytes, has led to the discovery a new form of cholesterol in the circulation: hemoglobin-lipid adduct termed Hb-Ch. The amount of Hb-Ch was found to be significantly higher in winter than in summer and to positively correlate with the plasma high-density cholesterol concentration. It was documented that consumption of dietary lipids, including saturated fat and cholesterol, has an important influence on the level of Hb-Ch in red blood cells. The low levels of Hb-Ch were found in the group of low-fat intake subjects compared with high-fat intake subjects. A high-lipid diet increased Hb-Ch in subjects with low Hb-Ch at onset, whereas a low-lipid diet decreased Hb-Ch in subjects with high Hb-Ch at onset. Efflux of cholesterol (and phospholipids) from Hb-Ch to fasting plasma high-density cholesterol fraction *in vitro* was also demonstrated. The results suggest that erythrocytes (via the Hb-Ch adduct) could provide a transient storage system for an excess of (potentially detrimental) un-esterified (exogenous) cholesterol in circulation. Hb-Ch neither increased autoxidation of hemoglobin to methemoglobin, nor it affected activity of anti-oxidant enzymes in red blood cells, indicating that the normal red blood cells are a safe place for storage of cholesterol.

Plant hemoglobins[30,31]

Less familiar to most people is the fact that hemoglobin(s) exist in plants. Three distinct types of hemoglobins have been discovered: symbiotic or leghemoglobins (Lbs), non-symbiotic (nsHb) and truncated hemoglobins (tHbs). These proteins have very high oxygen affinities because of an extremely low oxygen-dissociation constant.

First identified plant hemoglobins were from soybean root nodules. The leghemoglobins are monomeric proteins present in millimolar concentrations inside the root nodules of legumes. They are substantial component of the symbiotic nitrogen fixation machinery, facilitating oxygen diffusion from outside the root to the obligate aerobes involved in nitrogen fixation inside the nodule. This plant hemoglobin simultaneously fulfills the dual need for oxygen scavenging and transport, maintaining a low (10 nM) free oxygen concentration to prevent inhibition of the bacterial nitrogenase complex. Leghemoglobins regulate oxygen affinity through a different mechanism using a novel combination of heme pocket amino acids that lower the oxygen affinity. In contrast to hemoglobin and myoglobin, the presence or absence of the distal histidine has little impact on oxygen dissociation or affinity of leghemoglobin. Soybean leghemoglobin uses an unusual combination of B10 tyrosine and E7 histidine to prevent strong distal pocket stabilization of bound oxygen and the protein relies on a proximal mechanism to increase its oxygen affinity (20-fold greater than myoglobin).

The search for leghemoglobin homologs in non-legumes resulted in the identification of a class of "non-symbiotic" hemoglobins, believed to be present at low concentrations in all plants. The hexacoordinate hemoglobins, characterized by intramolecular coordination of the ligand binding site at the heme iron, were first identified in this hemoglobin type. Analysis of rice hemoglobin showed that distal histidine also coordinates heme ferrous atom and stabilizes bound oxygen. As a consequence, oxygen is not released easily from oxygenated nsHbs. Many of these proteins are upregulated by hypoxia or other stressors, suggesting functions additional to oxygen transport. In truncated hemoglobins (amino acid sequence 20-40 residues shorter than non-vertebrate hemoglobins), the abbreviated globin is porous, providing an almost continuous hydrophobic tunnel that may assist in oxygen binding to the heme, and the bound ligand is stabilized by more than one distal pocket residue. Characteristically, trHbs occur at nano- to micromolar intracellular concentration, hinting at a possible role as catalytic proteins.

Conclusion

New developments have forced a re-evaluation of our understanding of the structure and function of hemoglobin(s). Hemoglobin has essential functions besides carrying oxygen to the tissues, and regulates vascular tone and inflammation *via* a redox couple with methemoglobin. Hemoglobin has considerable pro-oxidant activity, especially outside the constraints of the erythrocyte and in the interactions with redox-active xenobiotic and metabolites**.** Hemoglobin glycation was suggested to induce oxygen-derived free

radicals causing oxidative damage to endogenous molecules, including cholesterol. On the contrary, cholesterol binding to hemoglobin has no effect on the sensitive redox balance in erythrocytes. Phylogenomic analysis showed that hemoglobin is widespread in living systems. Hemoglobin continues to play a spectacular role in the history and development of life sciences, with fascinating properties that still remains a challenge to its investigators.

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PON1 enzymes, analytical possibilities: ten years of experience

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Paraoxonase 1 (PON1) is a member of a 3-gene family localized on human chromosome 7q21-22. High-density lipoprotein-associated PON1 and PON3 are synthesized primarily in the liver, whereas PON2 is ubiquitously expressed in human tissues, membrane-bound, and may act as a cellular antioxidant[1]. Initial characterization and enzyme nomenclature was performed according to its ability to hydrolyze paraoxon, the toxic oxon metabolite of organophosphate compound parathion[2].

Paraoxonase-1 (PON1) is an organophosphate ester hydrolase (esterase) and lipolactonase which degrades lipid peroxides and is a part of antioxidant system of our body. PON1 level in systemic circulation is changed in a number of diseases involving oxidative stress as basic pathophysiological mechanism, such as cardiovascular disease, Alzheimer's disease, Parkinson's disease, chronic renal failure, HIV infection, metabolic syndrome, and chronic liver impairment[3]. Because of that PON1 investigation became more popular, but many analytical obstacles are not solved yet.

Introduction

High-density lipoprotein (HDL) is one of the key anti-atherogenic player at least in part due to its contribution to the reverse cholesterol transport process. HDL is also believed to protect against atherosclerosis by inhibiting the oxidative modification of low-density lipoproteins (LDL), thereby attenuating the biological activities of oxidised LDL[4]. Such anti-oxidant and anti-atherogenic properties of HDL have been attributed to various proteins associated with HDL, particularly the enzyme paraoxonase (PON). According to Aviram et al.[5], the anti-atherogenic properties of PON1 are characterised by its ability to hydrolyse lipid peroxides in human atherosclerotic lesions. Recent evidence has confirmed that PON enzymes are primarily lactonases acting upon certain lactones/hydroxyl acids as their endogenous substrates.

Therefore, one of the physiological roles of PON1 is the metabolism of lipid mediators arising from oxidation of polyunsaturated fatty acids[6]. Two of the many coding polymorphisms of PON1, the most frequent, are methionine (M) to leucine (L) substitution at position 55 and a glutamine (Q) to arginine (R) substitution at position 192. Both polymorphisms are associated with a number of pathophysiological conditions such as coronary artery disease (CAD), stroke, familial hypercholesterolemia, type 2 diabetes mellitus and Parkinson's disease[7]. In addition to those two polymorphisms, at least five other polymorphisms have been detected in the PON1 promoter region. There are substrate-dependent differences among *PON1Q192R* isoforms; the Q variant has a higher activity towards diazoxone (DZOase), whereas activity towards paraoxone (POase) predominates in the R variant [1].

Serum POase activity distribution studies in human populations revealed an activity polymorphism of high versus low POase activity. Studies on the polymorphic distribution of PON1 in human populations using a variety of different assays revealed bi or trimodal distributions of plasma POase activity[8].

By measuring the DZOase/POase activity ratios, it is possible to discriminate between the PON1Q192, PON1QR192, and PON1R192 activity phenotypes. Reports that seek to show an association between the *PON1192R* allele (or *PON1192RR* genotype) and cardiovascular diseases are controversial. There is also evidence that low PON1 activity is associated with atherosclerosis, irrespective of the PON1 phenotype[9].

According to Jarvik et al.[10] determination of this enzyme's activity level by using two substrate method is as important as genotype alone, because PON1 status determination gives us relevant information regarding susceptibility to disease, organophosphates sensitivity and some pharmacokinetic data. They stated that genotyping of polymorphic sites alone is insufficient to provide this important information but can be useful for gene frequency determination and forensic analysis.

PON1 analysis in Serbian population

We have performed this kind of PON1 status analysis on different groups of patients, whose disease is connected with atherosclerosis development[11-14].

In 2006. we have got our first results regarding PON1 activity phenotype, which were at the same time first results for the Serbian population regarding this enzyme phenotype and activity. Analysis were performed at the 261 middle aged subjects, 156 of which were coronary heart disease patients. This work establish ranges for the DZOase/POase ratio for the Serbian population, which separate subjects at one of the three PON1 192 phenotypes[11]. Namely, according to Richter and Furlong[15] the PON1192 phenotype should be predicted after examination of the two-dimensional plot of diazoxon vs. paraoxon hydrolysis rates. After initial plot examination we concluded that RR and QR phenotypes are not sufficiently separated, and we must used Adkins method for the different phenotypes separation[16]. We firstly calculated the DZOase/POase activity ratio and then determined the antimode of the histogram of this activity ratio. This work enabled us to assess DZOase/POase activity ratio for Serbian population, foor the first time. Individuals with the QQ phenotype have this ratio above 60, individuals with the QR phenotype between 21 and 59, and individuals with the RR phenotype below 20. (Figure 1).

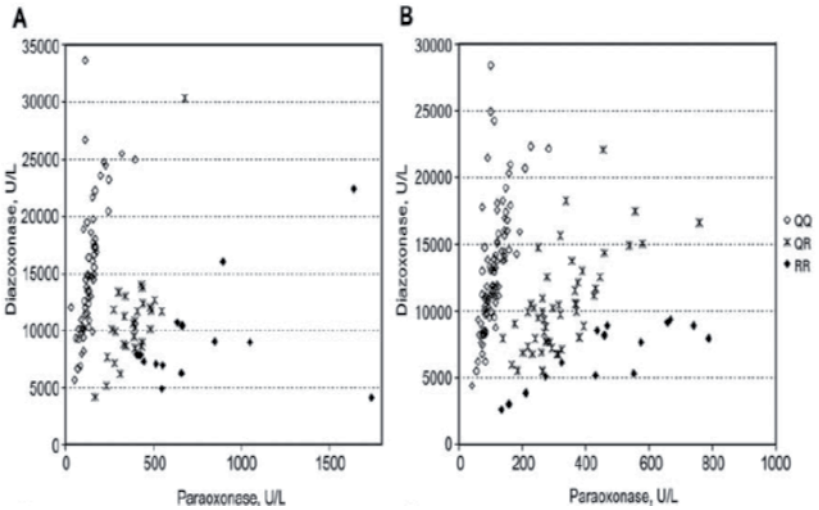

Figure 1. Population distribution plots of diazoxonase vs. paraoxonase activities in healthy people (A) and CHD patients (B) [11].

The PON1Q192R phenotype frequencies in 113 patients with occlusion larger than 50% (coronary artery disease-positive, CAD+ group) vs. control population were as follows: QQ (0.552 vs. 0.510), QR (0.382 vs.0.408) and RR (0.066 vs. 0.082); χ^2 =0.414, p=0.813. So we concluded that there were no difference in PON1 activity phenotype distribution between CHD patients and controls. We compared PON1 activities between healthy people and CHD patients in sub-groups according to their activity phenotype and found significantly lower PON1 activity in every phenotype subgroup in patients compared to controls (Figure 2). Our results regarding Serbian population confirmed data published by Jarvik et al.[10]. who have shown that PON1 activity was a better predictor of the disease than the PON1 genotype .

After this initial PON1 analysis we continued with the same analytics in several others groups of patients primarily in renal disease patients (children and adults) whose pathology is also connected with atherosclerosis development[12-14].

A significantly higher percentage of RR PON1192 subjects in the haemodialysis patient group compared to other groups (control vs. chronic kidney disease- CKD, renal transplant patients - Tx and haemodialysis patients - HD χ^2= 34.2, P< 0.001; HD vs. CKD, Tx χ^2= 27.01, P < 0.001) was evident [12].

Fifty-two pediatric chronic kidney disese patients were enrolled in the next study (10 with chronic renal failure [CRF], 22 with a renal transplant [RT], 20 with chronic hemodialysis (cHD) as renal replacement therapy, and 36 healthy children (control group, CG). We found lower POase activities in patients compared with controls . Patients also had lower DZOase activities, but they did not reach statistical significance. POase activity was lowest in the

cHD group (224 [162–298]U/L vs 490 [237– 679]U/L in the CG, p<0.038). Because of relatively small number of study subjects we didn't calculate activity phenotype distribution[13].

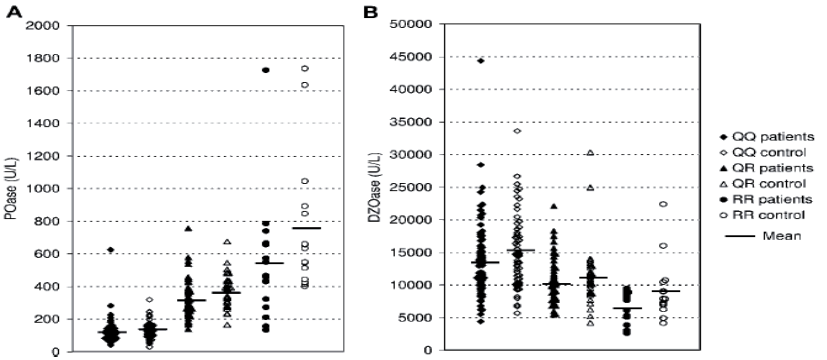

Figure 2. PON1 hydrolysis activity phenotype distribution in CHD patients and control according to three PON1 activity phenotype. POase activity panel A, DZOase [11].

The most recent study regarding PON1 status was on 185 acute ischemic stroke patients and 185 apparently healthy controls[14]. Results of this study showed significantly lower paraoxonase activity of PON1 enzyme in patients (an almost seven fold difference), compared with the control group. Stroke patients with lethal outcome had a higher risk RR phenotype frequency than in the CG (31% vs. 14%, $p < 0.05$. Stroke patients had significantly lower PON1 activity across all the PON1 (Figure 3).

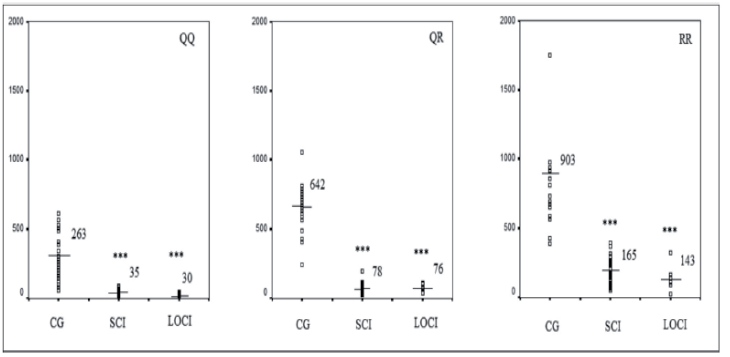

Figure 3. PON1 activity across PON1 192 activity phenotype in stroke patients (survivors cerebral insult – SCI and lethal outcome cerebral insult – LOCI) and control group (CG); ***P<0.001 vs. CG, numbers show mean values for the group[14].

Work with non-toxic substrates

Although paraoxone and diazoxone have excellent discriminatory capability to determine PON1 192 activity phenotype their toxicity, besides the fact that those two substances are not physiological PON1 substrates, are huge analytical problems. In order to solve this problem Richter et al.[17] have developed method determination of PON1 status with the use of non-toxic substrates. The two non-toxic substances are CMPA [4-(chloromethyl)phenyl acetate] and PA (phenyl-acetate) and graphical presentation of those two enzymatic activities CMPA vs. PA enable complete resolution of three activity phenotypes (QQ, QR, RR).

PON1 activity determination with physiological lactone type substrates

The physiological substrate or substrates for PON1 have not been identified. However, several lines of evidence show that this enzyme protects lipoproteins and cells from peroxidation by hydrolysing oxidised lipids. The available methods to measure serum PON1 activity use a variety of lactone-type substrates, such as 5-thiobutyrolactone (TBBL) and dihydrocoumarin[18]. PON1 polymorhism generally influences activity towards TBBL, although lower than paraoxon. Lactones are hydrolized preferentially by either PON1R or PON1Q depending on their structure; according to Gaidukov and coworkers[19] R isophorm has higher capability to hydrolyze lactone species.

Enzymatic assessment of paraoxonase 1 activity in different HDL subclasses on gel

Gugliucci and associates[20] developed practical method for analysis of PON1 enzymatic activity directly in HDL subclasses (HDL2 and HDL3), after their separation by gradient gel electrophoresis. It is well-known that HDL particles are heterogenic by size, so different HDL subclasses may have different atheroprotective capabilities. Which HDL fraction confers better cardiovascular protection remains controversial. According to some authors large HDL fraction has better atheroprotective capabilities, and the small HDL according to others[21]. Because of that it is extremely important to assess PON1 activity in distinct HDL fraction for every group of patients in whose disease this activity could be of importance. This methods uses reaction of PON1 on its substrate phenylacetate coupled with densitometric phenol detection using 4-aminoantipyrine. This method shed a light towards HDL functionality, which became more important than HDL-cholesterol concentration alone.

Conclusion

Although reliable methods for the routine measurement of serum PON1 may become available soon, at the moment we do not have neither definitive method, nor we know real physiological substrate. We need convenient methods with non-toxic, stable and more physiological substrates. The PON1 enzyme contributes to the antioxidative and atheroprotective functions of HDL, so further investigation of this intriguing protein is warranted.

Acknowledgements

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Root-induced changes at the soil-plant interface: from biochemistry to agriculture

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Besides the various functions (e.g. water and nutrient uptake, anchorage to soil), plant roots are able also to release a wide range of organic and inorganic compounds into the rhizosphere. Soil-chemical changes related to the presence of these compounds are important factors affecting availability of nutrients, solubility of toxic elements and thereby, the ability of plants to cope with adverse soil conditions. The aim of this review is to provide a comprehensive and updated overview of advances in the rhizosphere research, focusing on the mechanisms involved in release of protons and carboxylates by roots and its significance for nutrient availability and acquisition processes. In particular, I will highlight effects of the genotypic differences, plant physiological status and fertilization strategies on root exudation and its significance for increasing nutrient availability in the rhizosphere of various crop species.

Introduction

Plant roots are the organs for anchorage in soils and uptake of water and nutrients. Besides these functions, plant roots are able also to release a wide range of organic and inorganic compounds in the root-soil interface, called the rhizosphere. This term, coined for the first time by the German microbiologist Lorenz Hiltner in 1904, describes the volume of soil surrounding roots and affected by their activity[1]. Therefore, it is appropriate to state here that life on Earth is sustained by the only small volume of rhizosphere soil[2].

Plant roots can modify the rhizosphere chemistry in a number of ways: (a) by release of various organic compounds, (b) by gas exchange (CO_2/O_2) related with respiration of roots and rhizosphere microorganisms, and (c) by root uptake of nutrients associated with uptake or extrusion of protons and modifications of the soil pH and redox potential[3]. Soil-chemical changes related to the activity of living roots are important factors that influence microbial populations, nutrient availability for plant acquisition, solubility of toxic elements, and thereby, the ability of plants to cope with adverse soil conditions[4]. Most of these released compounds, called root exudates, are plant metabolites derived from photosynthesis and secondary metabolism. The stimulation of microbial activity and density in the rhizosphere is mainly due to the release of easily decomposable root exudates.

In this review, the root-induced changes in the rhizosphere and the mechanisms involved in release of protons and low-molecular weight (LMW) root exudates are described, and the role of these changes in plant nutrient acquisition from the rhizosphere is discussed.

Root-induced rhizosphere pH

The rhizosphere pH is important factor for plant availability of nutrients and toxic elements and may differ from the bulk soil pH by up to two units[4]. The most important factor for root-induced changes in rhizosphere pH is the uptake of nutrients, which is coupled with proton (H^+) transport through plant roots. The driving force for nutrient uptake by root pasmamembrane is H^+ extrusion, mediated by the activity of a plasma membrane-associated proton pumping ATPase (PM H^+-ATPse), which creates a gradient in electropotential and pH between the cytosol (pH 7.0-7.5) and the apoplast (pH 5.0-6.0)[4,5]. This electrochemical potential gradient is necessary for both proton-anion symport (H^+ uptake) and proton-cation antiport (H^+ release) types of nutrient transporters.

Uptake of cations and anions by roots is often not balanced[4]. Excess uptake of anions over cations leads to net removal of protons due to symport type of anion transport across the plasma membrane, thereby resulting in increased rhizosphere pH. In contrast, excessive uptake of cations is balanced by a net release of protons and consequently leads to rhizosphere acidification.

Nitrogen sources

Plants take up nitrogen (N) both in cationic (ammonium, NH_4^+) and anionic (nitrate, NO_3^-) forms. Nitrate is the main source of N for crop growth in well-aerated agricultural soils and nitrate is also more mobile in the soil than ammonium[3]. Nitrate uptake results in excess uptake of anions over cations, net uptake of protons and thus an increase in rhizosphere pH (Figure 1). The mechanisms by which nitrate is transported into the roots have been characterized both at physiological and molecular levels. Two high-affinity transport systems (i.e. constitutive, cHATS) and substrate-inducible, iHATS), encoded by *NRT2* gene family are involved in nH^+/NO_3^- symport at low nitrate concentrations in soil[6]. Therefore, this anion transport is an active process coupled to a H^+ electrochemical gradient generated by the PM H^+-ATPase. The expression of *HA1* and *HA2* genes coding for PM H^+-ATPase in roots is induced in the presence of nitrate[6]. In acid soils, the pH increase induced by nitrate supply enhances nutrient (e.g. P, Ca, Mg, Mo) availability and uptake and also decrease the availability of heavy metals and concentrations of toxic aluminium (Al) species[3, 5]. However, elevated nitrate supply may inhibit Fe uptake[7] and also decreases soil availability of Mn and Zn in neutral soils[3,5].

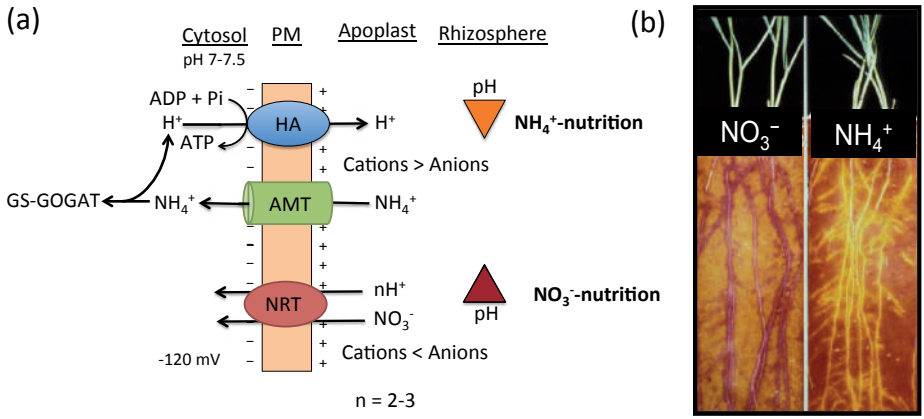

Figure 1. Root-induced changes in rhizosphere pH as affected by the form of N supply (a) and visualization of pH-changes by embedding of soil-grown wheat roots in pH indicator agar (b). Red, pH 6.5; purple, pH 7.5; yellow, pH 4.5. *Photo: Volker Römheld (by courtesy*).

In unfertilized agricultural soils, ammonium can be present in higher concentrations than nitrate. Ammonium uptake by roots is mediated by high-affinity transporters belonging to AMT1 subfamily, which transports ammonium via NH_4^+ uniport[3]. Once taken up by roots NH_4^+ is assimilated via glutamine synthetase-glutamate synthase (GS-GOGAT) pathway coupled with H^+ extrusion thereby decreasing the rhizosphere pH (Figure 1). In alkaline soils, the availability of P, Zn, Fe, Mn, Cu and B is very low, so ammonium-fertilizes can increase the nutrient availability. Ammonium-induced rhizosphere acidification may also increase the availability of heavy metals. Application of NH_4^+-based fertilizers has been proposed as a bioremediation strategy to improve the solubility and thus uptake of heavy metals in neutral and alkaline soils by accumulator plants (phytoextraction), while the pH remains high in the bulk soil preventing metal leaching[8].

Nutritional status

Root-induced changes in rhizosphere pH are also related to the nutritional status of plants. Examples are rhizosphere acidification in dicots under P and Fe deficiency[9,10]. One of the components of the so-called "Strategy I" root response mechanism to Fe deficiency in non-graminaceous plants is acidification of the rhizosphere via up-regulated HA family P-type H^+-ATPase (Figure 2). In neutral and particularly in alkaline soils, this serves not only to make rhizosphere Fe more available but also to facilitate optimal conditions for transmembrane reduction of Fe^{3+}-chelates via expression of a PM-bound reductase oxidase

(FRO family) with a low pH optimum (Figure 2). Highly localized acidification in apical root zones (sites for Fe^{3+} reduction and subsequent uptake of Fe^{2+} via the IRT transporter, which is up-regulated under Fe limitation) of Fe efficient plants species and genotypes may enable the roots to decrease the rhizosphere pH even in well-buffered calcareous soils.

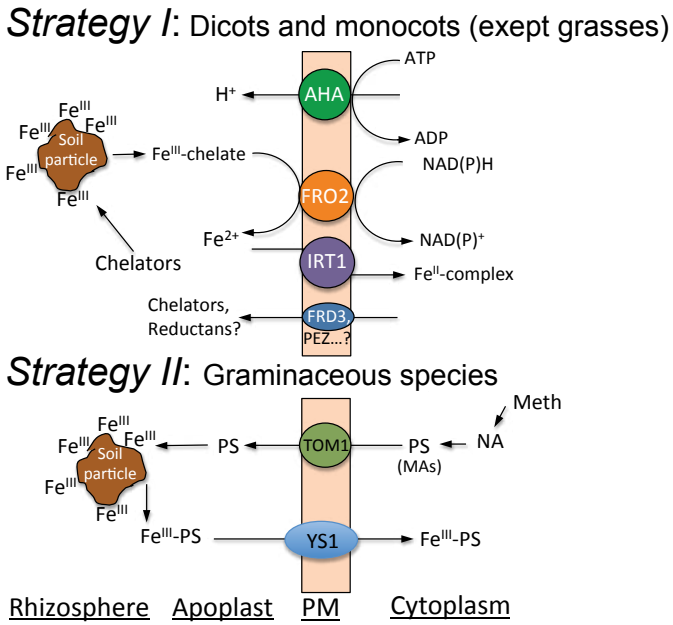

Figure 2. Model for Fe deficiency-induced changes in root physiology and rhizosphere chemistry associated with Fe acquisition in Strategy I plants in Strategy I and Strategy II plants. PS, phytosiderophores; MA, mugineic acid; NA, nicotianamine.

Excess uptake of cations in some dicotyledonous plant species such as tomato, chickpea, and white lupine is balanced by enhanced net H^+ release provided by increased biosynthesis of organic acids via glycolysis and phophoenolpyruvate carboxylase (PEPC) as a constituent of the intracellular pH-stat mechanism (for detail see the next section). In these plants, P deficiency-mediated H^+ extrusion via up-regulated PM H^+-ATPase leads to rhizosphere acidification, which can contribute to the solubilization of acid-soluble Ca-phosphates in calcareous soils (Figure 3a). When N_2-fixing legumes are grown together with non-legumes (e.g., faba bean/maize intercropping), rhizosphere acidification of legumes can increase P acquisition by non-legume crops[11,12].

Mechanisms and control of root exudation

Phosphorus deficiency and Al toxicity in acid soils

When grown on acid soils, most crop species exhibit characteristic adaptive strategies including the exudation of carboxylates, phenolics and flavonoids[13,14]. Since P deficiency and Al-toxicity coexist in acid soil[15], the secreted organic compounds can mobilize P from immobile P complexes and also chelate toxic Al^{3+} ions in the rhizosphere (Figure 3b). Exudation of organic anions (mainly malate and citrate) from plant roots in response to Al toxicity is well documented although their effect on P acquisition has not been sufficiently studied[16]. So far, the extent in which either Al toxicity or P deficiency triggers the release of carboxylates by roots on acid soils remains unclear. Malate and citrate have different exudation patterns, and the release of both is controlled by the organic anion transport proteins belonging to an Al-activated malate transporter (ALMT) family and/or to a multidrug and toxic compound extrusion (MATE) family[17,18]. In many Al-tolerant plant species and cultivars, Al-induced root secretion of carboxylates (particularly malate, citrate and oxalate) is an important factor for Al^{3+} detoxification by external complexation in the root apoplast[16]. Some members of the ALMT and MATE families mediate carboxylate transport independently of Al stress. For instance, it has been shown that wheat *TaALMT1* can also be overexpressed under low soil P conditions and can mediate ion transport in the absence of Al^{3+} in soil solution[19,20].

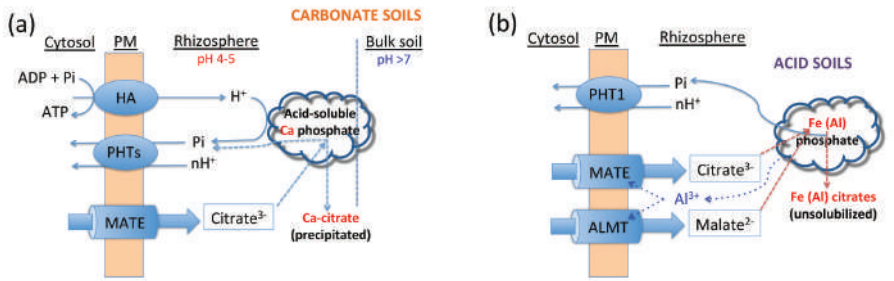

Figure 3. Model for P deficiency-induced changes in root physiology and rhizosphere chemistry associated with P acquisition in carbonate (a) and acid (b) soils.

Liming (application of $CaCO_3$) is a common measure for acid soils in agricultural practice. Its main amending effect at the rhizosphere level is amelioration of soil constraints other than low P availability (e.g., rhizotoxicity of H^+ and Al^{3+}) and hence recovering P acquisition potential of roots. Recently, Kostic et al. demonstrated that P-deficient wheat roots not subjected to Al stress in the limed soil can maintain high efflux of malate and even increase efflux of citrate along with the enhanced expression of *TaMATE1* and *TaALMT1* genes[20].

Also, application of Si-based fertilizer increased exudation capacity of citrate and malate for P mobilization in the rhizosphere, along with an increased expression of TaMATE1 and TaALMT1 transcripts in wheat plants grown under low P conditions[21,22].

Enhanced expression of glycolytic enzymes and PEPC represent a widespread metabolic modification in P-deficient plant tissues[3]. The cytosolic PEPC catalyzes the carboxylation of phosphoenolpyruvate (PEP) to oxaloacetate (non-photosynthetic CO_2 fixation), which can be further converted to malate by enhanced expression of cytosolic malate dehydrogenase[3, 5]. Therefore, PEPC may operate as an alternative bypass of carbohydrate catabolism under P-deficient conditions, which facilitates a more economic Pi utilization than Pi liberation from PEP via pyruvate kinase, and also enhances anaplerotic biosynthesis of carboxylates for more efficient P mobilization in the rhizosphere (Figure 4).

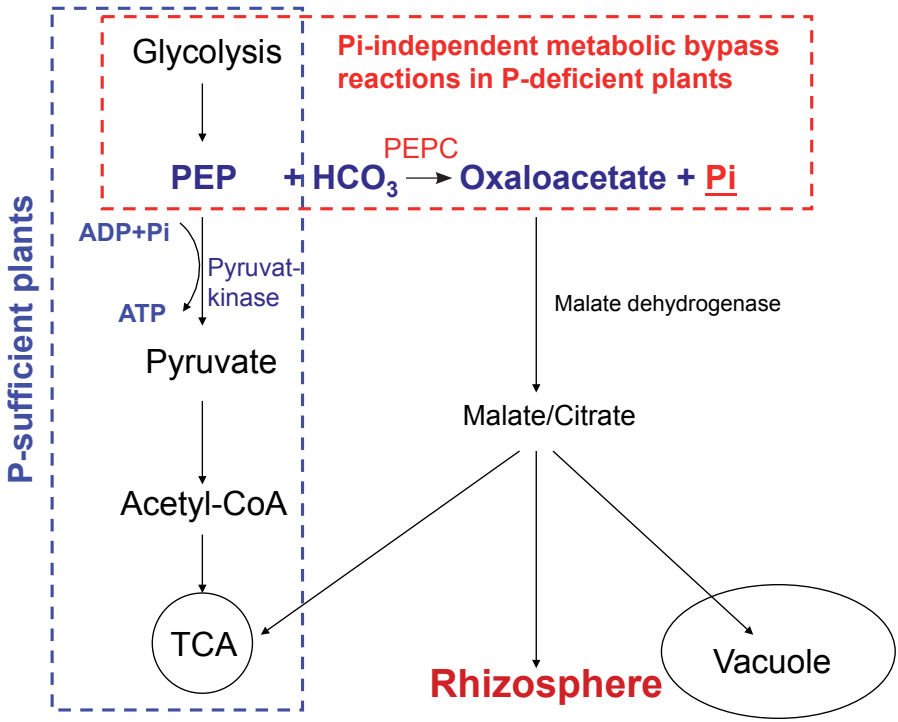

Figure 4. Model for P deficiency-induced biochemical changes associated with the release of P-mobilizing root exudates in roots.

Iron and zinc deficiency in calcareous soils

Under Fe starvation, many Strategy I species (dicots and non-gaminaceous monocots) enhance root accumulation and thus exudation of organic compounds such as carboxylates, phenolics and flavins, which can increase Fe availability due to chelation and potential non-enzymatic reduction of Fe^{3+} (Figure 2). In graminaceous plant species, so-called "Strategy II" plants (Figure 2), the root biosynthesis and release of natural metal chelators, such as the mugineic acid (MA) family of phytosiderophores (PSs) is induced by limitation of Fe and Zn[3, 23]. In the rhizosphere, PS mobilize Fe^{3+}, but also other micronutrients, such as Zn, Mn and Cu, by formation of stable complexes even at high soil pH. There are large genotypic differences in the capacity for PS secretion between plant species and cultivars (e.g. barley >rye>wheat>maize>sorhum>rice)[23]. Phytosiderophores are synthesized at high rates in the roots of Fe- and Zn-deficient graminaceous plants from a precursor nicotianamine (NA), which is an ubiquitous intracellular metal chelator in higher plants. This biosynthesis is regulated by nicotianamine aminotransferase (*NAAT*) gene[23]. The soluble Fe^{3+}-PS complex is subsequently taken up via specific transporters belonging to yellow stripe-like (YSL) family of proteins. Up-regulation of *YS1* is induced by Fe deficiency, but not by deficiency of other micronutrients such as Zn, Mn or Cu[23].

Various authors suggested the use of grasses as cover crops to prevent or cure Fe-chlorosis in crop fields, orchards and vineyards as more sustainable and environmental benign measure[24–27]. It has been proven that PS released by graminaceous species can mobilize Fe from sparingly soluble soil sources and the Fe^{3+}-PS complexes thus formed can be taken up by dicotyledonous plants, which explains the beneficial effects of intercropping dicots with grasses (Figure 5).

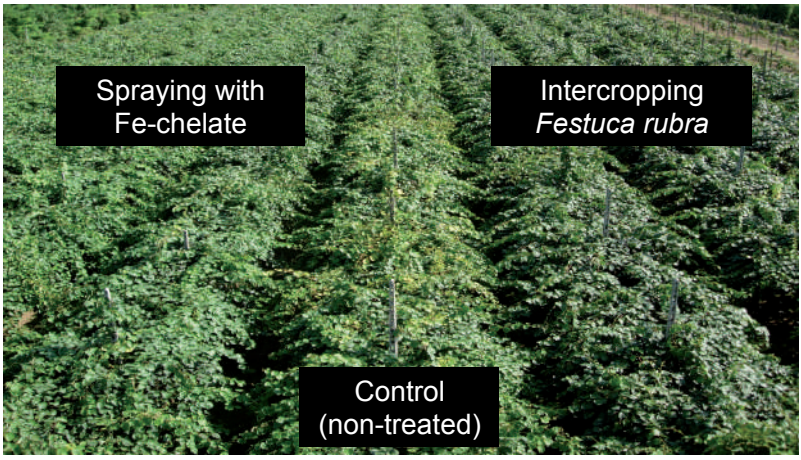

Figure 5. Practical application of the rhizosphere knowledge: intercropping successful in remedy of Fe chlorosis in a vineyard (Emilia-Romagna, Italy). *Photo: Adamo Domenico Rombola (by courtesy).*

Concluding remarks and perspectives

Manipulation of the root biochemistry (e.g. release of protons and LMW root exudates) in crops by use of genetic engineering, breeding technologies or modification of the rhizosphere management and cultivation systems, to increase the efficiency for nutrient acquisition, the resistance to adverse soil-chemical conditions has become more globally accepted in agricultural practice. This requires a detailed knowledge of the physiological and biochemical mechanisms involved in the regulation of root exudation and of the overall rhizosphere processes. During the past decade rapid progress has been made in the characterization of regulatory processes involved in root exudation at the molecular level. Therefore, a better understanding of the adaptive functions and the behavior of root exudates in the rhizosphere and the control of release mechanisms may directed selection and breeding strategies for plant genotypes adapted to adverse soil conditions and likewise foster the improvement of crop rotations and intercropping systems.

Acknowledgements

This paper is dedicated to the memory of Professor Volker Römheld (Stuttgart-Germany).

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Molecular regulation of insect diapause

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Diapause is a well-regulated, complex and dynamic state of arrested development which consists of several successive and overlapping phases. Even though mechanistic nature of diapause varies among different organisms, there are many shared biochemical and molecular processes that govern this type of dormancy. Here, in this paper, selected molecular hallmarks of diapause are presented, based on the studies on the European corn borer, *Ostrinia nubilalis*, a model organism for winter diapause in insects.

Introduction

In order to avoid environmental atrocities, including cold temperatures, low oxygen, lack of food or water, numerous organisms have evolved the ability to enter into dormant phase until the favourable conditions return[1]. For instance, insects of temperate and subpolar zones have developed an ability to enter into diapause, a state of developmental and metabolic arrest. Diapause occurs at different developmental stages, but usually only at one particular life history stage of a given insect species[1]. Obligatory diapause is genetically controlled part of a life cycle that is internally driven and occurs regardless of environmental adversities. On the contrary, facultative diapause occurs in an opportunistic manner, for it is induced by various environmental stimuli[2]. Diapause should not be regarded as a passive state, but rather as a dynamic, well-regulated and complex process that consists of several successive and overlapping phases – induction, preparation, initiation, maintenance, termination, and post-diapausal quiescence[3].

Though mechanistic nature of diapause varies among different organisms, there are many shared biochemical and molecular mechanisms that govern the entry into and transition through the diapause[4]. Selected hallmarks of diapause include: a gradual accumulation of metabolic reserves, metabolic rate depression, reductions in energy expenditure, differential gene expression, prolongation of mRNA half-life, post-translational protein modifications and biosynthesis of protective compounds[3,4,5]. Further, as a state of arrested development and prolonged hypometabolism, diapause is inevitably associated with various stressful challenges — thermal stress, dehydration, impairment in energy production, increased production of ROS/RNS, reduction in the activity of ion pumps and subsequent ion leakage[1,4,6,7,8,9,10]. Thus, all molecular, physiological, morphological and behavioral changes in a given organism synergistically increase its overall stress tolerance[1].

Selected metabolic alterations

It is widespread that organisms depress their metabolic rates in order to survive prolonged period of environmental stress[11]. Metabolic rate is depressed to lower levels than of resting metabolism and such a depression is the best way to preserve limited amounts of internal energy supplies, which need to be carefully exploited during days, months and sometimes even years of dormant period. Cellular respiration is the central bioenergetic process and enzymes involved in it must be carefully coordinated to ensure an efficient energy production in the cell. For this reason, change in the kinetics of these enzymes may seriously disrupt cellular homeostasis. Mitochondria are in the center of oxygen-based metabolism and almost all oxygen in cells is consumed by cytochrome c oxidase (complex IV, COX). Thus, reduction in O_2 consumption during dormancy is of vital importance and is achieved by decreasing the number of mitochondria and/or reducing the activity of COX. In diapauses of European corn borer, *Ostrinia nubilalis*, the activity of COX as well as the expression of *cox1* are suppressed in the first part of diapause (Figure 1. A, B)[12].

Moreover, for a long term homeostasis in dormancy, a careful balance between the ATP-generating and ATP-utilizing processes is imperative. Reorganization of metabolic priorities includes suppression or complete exclusion of unnecessary energy-consuming process and maintenance of processes critical for survival[4]. For instance, since the ATP-driven ion pumps represent the most prevalent energy-consuming process in the cell, metabolic arrest in dormant phase must include mechanisms for reducing the overall rates of ion cycling. However, due to their key role in maintenance of membrane potential, the activity of ion pumps is preserved in dormancy at low, but energetically sustainable level.

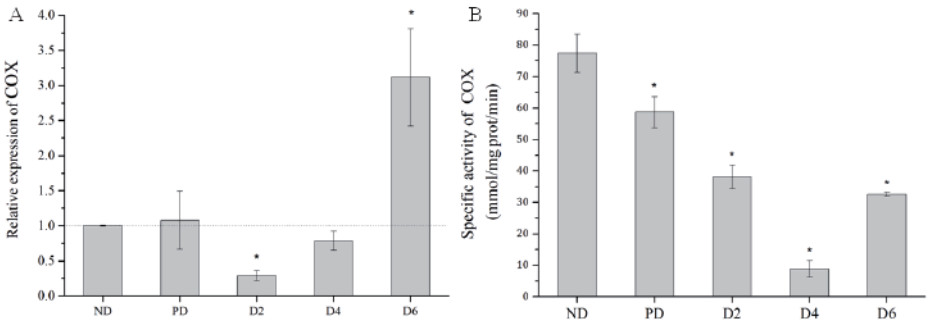

Figure 1. Relative expression (A) and specific activity (B) of COX in diapause of *O. nubilalis*[12]. ND-non–diapausing, control group, PD – prediapausing, D2 – two months in diapause, D4 – four months in diapause and D6 – six months in diapause.

Since, the number of ATP-utilizing processes is higher than the number of ATP-generating processes, in general, level of ATP decreases during dormancy while in the same time ADP and AMP increase (Figure 2). Such changes in the concentration of adenine nucleotides in the cell usually lead to the decrease in energy charge, like in the winter diapause of *O. nubilalis* (Figure 2)[12]. Products of ATP degradation have substantial impacts on cellular metabolism since they act as regulatory molecules that sense energetic status of a given organism. Namely, AMP is a potent allosteric regulator of various enzymes of which AMP-activated protein kinase (AMPK) is the most prominent[4,8], since it regulates a number of cellular processes.

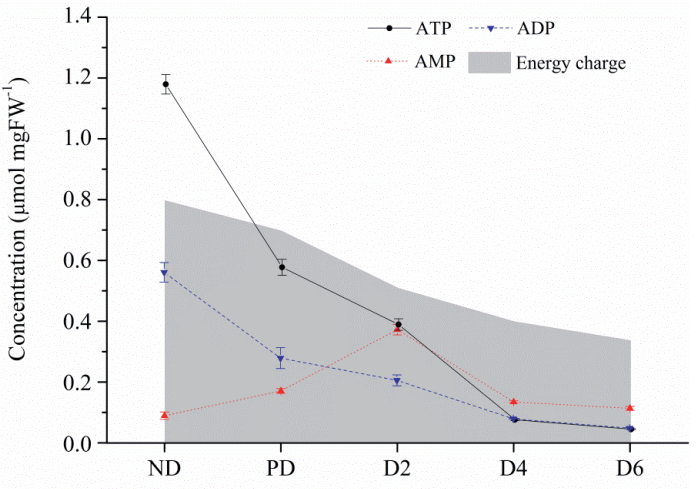

Figure 2. Relative expression (A) and specific activity (B) of COX in diapause of *O. nubilalis*[12]. ND-non–diapausing, control group, PD – prediapausing, D2 – two months in diapause, D4 – four months in diapause and D6 – six months in diapause.

Hypoxia and metabolic stresses, which alter ATP synthesis, trigger AMPK activity[1]. AMPK is a heterotrimeric enzyme, consisting of catalytic α-monomer and regulatory β and γ subunits that are encoded by different, usually multi copy genes. AMP binds to γ regulatory subunit, while β subunit posses a glycogen binding domain, which interestingly may also serve as an energy sensor during diapause. In cells of dormant organisms, AMPK suppress unnecessary energy-consuming biosynthetic pathways, such as protein synthesis, turns on alternative ATP-producing catabolic pathways and inhibits cell cycle by activating p53 and p27[1,7,11]. In general, reversible protein phosphorylation (RPP) is widely accepted mechanism which governs metabolic reorganization in dormancy. Apart from AMPK, other proteins kinases proved to be involved in metabolic alterations in dormancy – PKC, PKG, MAPK[4,7,11]. Reversible phosphorylation of existing proteins seems to be faster, energetically favourable and more efficient mean of metabolic regulation in dormancy than synthesis of new regulatory proteins[11].

Differential gene expression

It is estimated that 1-25% of cellular energy is devoted to transcription and translation[5]. Thus, suppression of transcription and translation in dormancies is expected and widely documented in numerous organisms. Modes of transcriptional suppression are numerous and range from histone modifications and regulation of RNA polymerase II to post-transcriptional inhibitory action of short non-coding regulatory RNAs[1,5,8]. However, even though general protein synthesis is strongly suppressed in dormancy, up-regulation of genes coding for products involved in cellular homeostasis maintenance and stress protection is well-documented in all analyzed dormant species[1,4]. Differential gene expression during diapause is described in numerous studies with diverse methodologies applied[1]. Such studies created a vast list of mRNAs and proteins that prove to increase/decrease or are stable during diapause[1,7]. Still, most of such studies failed to demonstrate function of analyzed genes, since only levels of mRNAs and/or proteins were measured. However, generated data is nonetheless very informative for it comments previous studies as well as opens new perspectives for the subsequent investigations. Most of the studies on gene and protein expression during diapause in insects have documented the upregulation of HSPs and other protective proteins[1]. Similarly, in a study on diapause in *O. nubilalis*[10], differential expression of four distinct HSPs was documented using Real Time PCR – HSP90, Hsc70, HSP20.4 were upregulated, while HSP20.1 was downregulated in different periods in diapause (Figure 3).

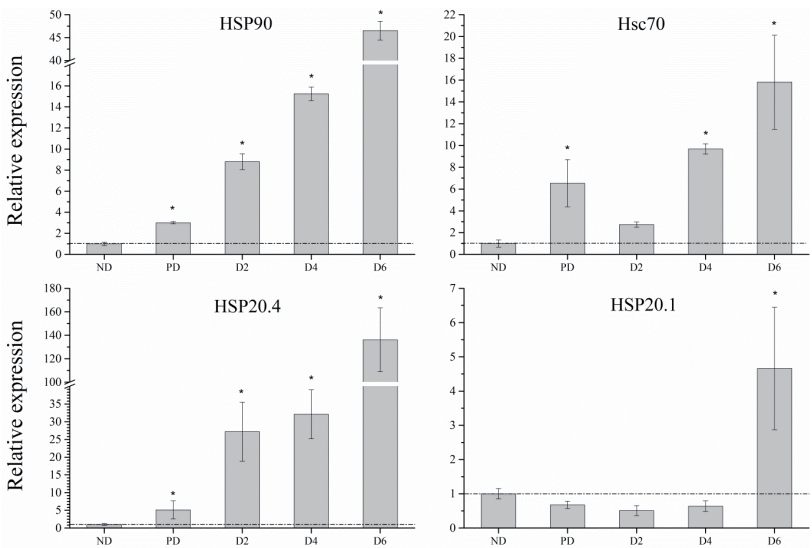

Figure 3. Relative expression of selected HSP genes in diapause of *O. nubilalis*. ND-non–diapausing, control group, PD – prediapausing, D2 – two months in diapause, D4 – four months in diapause and D6 – six months in diapause.

Similarly, the expression of genes coding for ferritin and metallothionein, proteins involved in homeostasis of metal ions, was increased in dormancy of *O.nubilalis* (Fig. 4). Expression of ferritine and metallothionein may serves to prevent bacterial infections and decrease in ROS production as well as to increase tolerance during cellular dehydration[10,12].

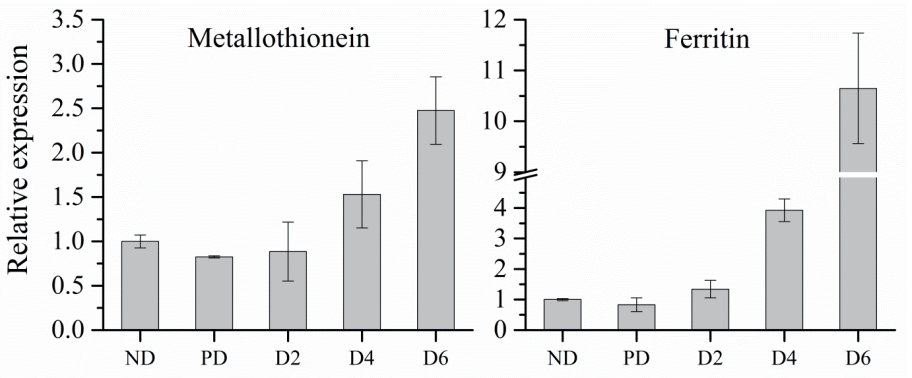

Figure 4. Relative expression of metallothionein and ferritin in diapause of *O. nubilalis.* ND-non–diapausing, control group, PD – prediapausing, D2 – two months in diapause, D4 – four months in diapause and D6 – six months in diapause.

Furthermore, results in this study have shown that both diapause programming and temperature of acclimation influence the expression of genes coding for products related to stress tolerance. A progressive increase in the relative abundance of analysed mRNAs during diapause probably reflects the impact of low temperatures on both the expression of genes and the stabilisation of mRNAs, which have been documented in dormancies before[1,7]. Also, cold temperatures not only may upregulate genes but also may suppress the activity of RNases, stabilise and prolong half-life of mRNAs, which leads to a gradual accumulation of transcripts during diapause[10].

General remarks

Diapause is widely exploited among different animal groups ranging from nematodes to mammals. It is accompanied by a plethora of molecular, biochemical and physiological changes that mutually enhance stress survival and synchronize reproduction[1,5]. Even though mechanistic nature of diapause substantially vary among different species, many shared biochemical and molecular hallmarks have been identified and include gradual accumulation of metabolic reserves ahead of diapause, metabolic rate depression, reduction of energy expenditure, lipid reorganization in membranes, differential gene expression, prolongation of mRNA half-life, post-translational protein modifications and biosynthesis of protective

compounds [1,3,5,10,11,12]. Still, many important questions regarding diapause remain incomprehensible such as – What are the molecular mechanisms regulating cell fate e.g. the cell cycle and growth arrest as well as the suppression of cell death? Also, whether these mechanisms are similar from species to species? How are sets of diapause-specific genes regulated? What are molecular switches inducing transition from active to dormant metabolic state?

Diapause should remain in the focus of study, since better understanding of biological and ecological nature of this type of dormancy may lead to the improvement of field control of pest species, insect rearing techniques in laboratory as well as may serve as a potential rich source of new pharmaceuticals, due to the altered metaboloms in dormant organisms[13].

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Evaluation of CXCR4 receptor and FAK kinase as possible targets for non-small cell lung carcinoma treatment

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Non-small cell lung carcinoma (NSCLC) is the most common type of lung cancer. Death rate of this disease is very high mainly due to development of metastasis. In large group of molecules involved in metastatic processes CXCR4 receptor and focal adhesion kinase (FAK) stand out as particularly important and interesting. Their role in pathogenesis of NSCLC was extensively studied in last decade. Both CXCR4 and FAK were proved to have abnormally high expression in significant portion of NSCLCs and to associate with increased metastatic potential and poor prognosis. Their crucial role in adhesion, migration and invasion of NSCLC cells was also confirmed in *in vitro* and *in vivo* studies. Moreover, several studies have shown that CXCR4 and FAK were altered in response to chemo- or radio-therapy. Therefore, all these data imply that CXCR4 and FAK could be considered as promising targets for NSCLC treatment. Accordingly, blocking either of these two molecules was evaluated in several studies but this area of research requires further detailed examinations particularly regarding their relationships with conventional therapeutic approaches in NSCLC.

Introduction

Cancer is, in addition to cardiovascular and infectious diseases, major health problem of the modern world. Among all cancer types, lung cancer is the most common, with the highest incidence of new cases (1.8 million cases, 12.9% of total) and deaths (1.6 million deaths, 19.4%) and with an overall five-year survival rate less than 15%[1, 2].

Histologically, lung cancer is comprised of four major types: adenocarcinomas, squamous cell carcinomas, large cell carcinomas and small cell lung carcinomas. Based on the clinical prognosis and response to therapy adenocarcinomas, squamous cell carcinomas and large cell carcinomas are classified into a single group of non-small cell lung carcinomas (NSCLC) which accounts for approximately 75% of all lung cancers[3].

Current treatment protocols for NSCLC patients involve administration of chemotherapy along with surgical resection for early stage NSCLC patients and radiotherapy in the treatment of patients with late-stage NSCLC[2]. However, conventional chemotherapy often fails to eliminate all the cancer cells, at least partially, due to dissemination of tumor cells through the body. Most of the lung cancer patients at the time of diagnosis already have symptoms of advanced disease with metastases. Even at the earliest stage of the lung cancer substantial percentage of patients already have micrometastases[4] and it was shown that patients with metastases have increased level of serum free-DNA that significantly correlates with response to therapy[5]**.** Therefore, targeting molecules that are involved in migration and invasion of cancer cells could be one promising approach in improving chemotherapeutic efficacy and survival in lung cancer.

One of the important molecules that contribute to cell's migration and invasion is CXCR4. CXCR4 is chemokine receptor that is activated by chemokine CXCL12, previously called stromal cell-derived factor-1 (SDF-1). It is seven-transmembrane G-protein-coupled receptor (Figure 1.) that recognizes the cysteine rich domain, CXC, of CXCL12 chemokine which is its only ligand. The CXCR4/CXCL12 axis is important regulator of cell trafficking and homing to target sites that highly express respective ligand. These processes are essential during development, and for homeostasis and function of the immune and stem cell systems. It was initially cloned from leukocytes and shown to be target for Human Immunodeficiency Virus (HIV) binding and entry into cells. However, recently data implied that CXCR4 was highly expressed in number of human malignancies such as acute and chronic leukemia, breast cancer, prostate cancer and lung cancer. In human cancers, excessively activated CXCR4 promotes tumor cell survival, proliferation, invasion and metastasis[6, 7].

One of the molecules that is activated upon stimulation of CXCR4 is focal adhesion kinase (Figure 1). FAK is cytoplasmic non-receptor tyrosine kinase recognized as the key mediator of growth factor receptors and integrin signaling. It is involved in regulation of number of important cellular processes such as: adhesion, spreading, motility, invasion, metastasis, survival, angiogenesis and epithelial to mesenchymal transition (EMT). In addition it is vital for maintenance of cancer stem cells and tumor microenvironment. This kinase is overexpressed in most of the solid tumors as well as in non-solid malignancies. Similarly to CXCR4, FAK is highly expressed in lung cancers, including NSCLC[8, 9].

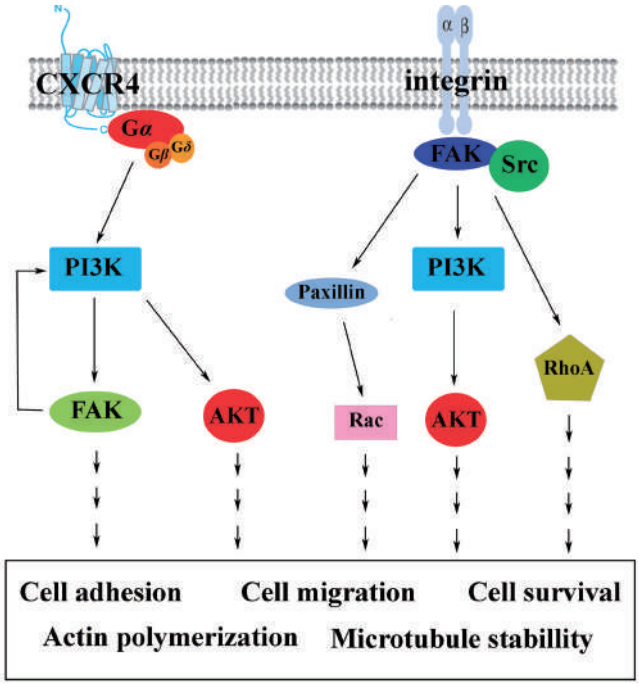

Figure 1. CXCR4 and FAK signaling scheme.

Role of CXCR4 in NSCLC

Significant role of altered expression of CXCR4 in pathogenesis of NSCLC was recognized recently and studying this molecule in NSCLC progression has particularly came into focus in last few years. Latest meta-analysis has shown that CXCR4 expression was significantly higher in NSCLC samples than in normal lung tissue[10]. Phillips et al. showed that CXCR4 is highly expressed in both squamous cell carcinoma and adenocarcinoma subtypes[11], while according to study of Imai and colleagues its overexpression is significant characteristic of patients with adenocarcinoma as well as females and non-smokers[12]. Even though this chemokine receptor is highly expressed in tumors of all stages relative to normal lung samples[11, 13, 14], its expression was significantly higher in advanced than in early staged NSCLC[10]. Moreover, as expected from its cellular function, CXCR4 overexpression significantly associates with tumor invasion in lymph nodes as well as presence of distant metastases[10]. It was shown that high CXCR4 expression correlates with brain specific NSCLC metastases[15-17]. High expression of CXCR4, on protein and mRNA level, associates also with poorer overall patient survival[10, 18]. Interestingly, effects of CXCR4 expression depend on its cellular localization. Nuclear staining of CXCR4 associates with adenocarcinoma,

advanced tumor stage, lymph node invasion, and distant metastases. On the other hand, nuclear localization significantly contributes to prolonged overall and disease free survival, while cytomembranous staining coincide with poor prognosis[18, 19].

All these data, obtained on patient samples regarding the role of CXCR4 in NSCLC pathogenesis, were confirmed using *in vitro* and *in vivo* model systems. Philips et al. showed that NSCLC cell lines, both adenocarcinoma (A549) and squamous cell carcinoma (Calu-1) cells, constitutively expressed CXCR4 mRNA with high presence of the protein on cell surface[11]. They also revealed that CXCL12/CXCR4 axis does not influence cell proliferation nor apoptosis but is rather responsible for the promotion of chemotaxis. The cells with higher CXCR4 mRNA level and protein expression on cell membrane showed increased metastatic potential[20]. *In vivo* studies also pointed to the implication of CXCR4 in NSCLC metastasis. Specifically, Su et al. showed that inoculation of lung cancer cells with low expression of CXCR4 in nude mice showed up to 2-fold decrease in lung metastatic foci than that with high expression of CXCR4[20]. Similarly, in experiments on SCID mice, Phillips et al. demonstrated that distant NSCLC metastases had higher percentage of CXCR4 positive cells than primary tumor[11].

Targeting CXCR4 in NSCLC

Considering important role of CXCR4 in progression and dissemination of NSCLC its targeting could have great significance in treatment of NSCLC. So far, blocking CXCR4 activity was primarily used for more detailed elucidation of its function in this type of cancer. Therefore, several different approaches have been exploited to date to inactivate CXCR4 and they included use of: 1) receptor antagonists, 2) neutralizing antibodies, and 3) small interfering RNAs (siRNAs).

Application of CXCR4 specific antagonists was most thoroughly examined method for abolishing its functions. AMD3100 (originally called JM3100 and recently renamed as Plerixafor or Mozobil™) is most commonly used CXCR4 antagonist. It is a bicyclam, in which two cyclam rings are linked through an aromatic bridge. This drug was approved by the US FDA for mobilizing hematopoietic stem cells to the peripheral blood for collection and subsequent autologous transplantation in patients with non-Hodgkin's lymphoma and multiple myeloma, as well as for leukemia patients to mobilize leukemia cell and subsequently target them by conventional drugs[7]. However, AMD3100 is still in preclinical evaluation process for treatment of NSCLC. In *in vitro* studies this CXCR4 inhibitor, as well as CXCR4-neutralizing antibody and small interfering RNA against CXCR4, were efficiently exploited to show that CXCL12 induced the migration of lung cancer cells through CXCR4 signaling that ultimately regulates MMP-9 transcription and gelatinase activity[21, 22]. In addition, Jung et al. succeeded to suppress sphere forming activity in stem like A549 gefitinib-resistant cells by application of AMD3100 or CXCR4 specific siRNA[23], while Wald et al. efficiently used the same inhibitor to prevent CXCL12 induced colony formation in different NSCLC

cell lines[24]. Recently, Singla and colleagues showed that AMD3100 treatment did not affect number of metastases but suppressed the growth of those metastatic foci in murine model of metastatic human non-small cell lung cancer[25].

Another CXCR4 antagonist, BKT140 (Biokine), that showed strong mobilization of hematopoietic stem cells, was investigated for its effects in NSCLC. Fahham and colleagues demonstrated that this small peptide reduced colony forming of various NSCLC cell lines and inhibited their proliferation in both cytotoxic and cytostatic manner[26]. Moreover, they showed that BKT140 delayed tumor growth and decreased tumor size *in vivo*.

Similarly, Peng and co-workers showed that LY2510924 is potent CXCR4 antagonist that abrogates CXCL12/CXCR4 signaling and efficiently inhibits tumor growth of A549 tumor xenograft model[27]. LY2510924 is a small cyclic peptide containing non-natural amino acids which is currently in phase II clinic study for metastatic clear-cell renal cell carcinoma.

As already mentioned, CXCR4-specific siRNA technology and neutralizing antibodies were used, efficiently as CXCR4 antagonists, in number of studies to suppress CXC12/CXCR4 signaling and prove its role in migration and invasion of lung cancer cells[20-22, 28].

Except reducing metastatic potential, targeting CXCR4 might be also used in preventing and overcoming resistance to chemo- and radiotherapy. Recent study revealed that chemotherapy, specifically paclitaxel and carboplatin, could enhance metastasis due to increased level of cytokine and angiogenic factors, including CXCR4[29]. Additionally, CXCR4 was shown to contribute to gefitinib resistance in A549 NSCLC cell line[23]. Another study demonstrated that irradiation of NSCLC cells significantly upregulated CXCR4 expression at both the mRNA and protein levels and increased their invasiveness via the CXCL12/CXCR4 pathway[30]. Overall, it seems rational that several authors combined application of CXCR4 inhibitors with conventional cytotoxic drugs or radiotherapy in NSCLC treatment. Namely, two research groups used LFC131 peptide, linear type of low molecular weight CXCR4 antagonist, conjugated nanoparticles to deliver doxorubicin or docetaxel in targeted and controlled manner to A549 cell that overexpress CXCR4[31, 32]. On the other hand, Fahham et al. applied BKT140, CXCR4 antagonist, along with paclitaxel, cisplatin or radiation to treat NSCLC cell lines and obtained an additive antiproliferative effect of these combined treatment protocols[26]. Finally, Gu and colleagues achieved to suppress irradiation-induced invasiveness by AMD3100 inhibitor[30].

Role of FAK in NSCLC

FAK is another molecule that is, due to its important role in cell migration and invasion, extensively studied in number of human cancers. This kinase is significantly overexpressed in NSCLC compared to normal lung tissue and its high tumor activity associates with advanced disease stages and lymph node metastasis[33, 34]. Even though there are no significant differences in FAK expression between adenocarcinoma and squamous cell carcinoma,

it is up-regulated in both subtype samples and significantly related to histopathological parameters indicators of advanced disease with metastases in each subtype[33-35]. In addition, overexpressed FAK significantly decrease NSCLC patients' survival, as well as survival of those with adenocarcinoma subtype, and represents independent factor for predicting poor prognosis in NSCLC[34, 36]. *In vitro* studies have shown that FAK is important component of extracellular matrix (ECM)/integrin-mediated signaling pathways and that it is stimulated upon cellular interaction with fibronectin or collagen, further promoting migration and invasion of A549 cells[37, 38].

Targeting FAK in NSCLC

Direct targeting of FAK was far less exploited in NSCLC than in other tumors. Silencing with FAK specific siRNAs in NSCLC cell lines showed reduced phosphorylation of FAK protein with subsequent decrease in colony formation and migration but with no influences on cell apoptosis[39]. Similar results were obtained with antisense oligonucleotides, as well as with FRNK (FAK related non kinase), which is autonomously expressed, noncatalytic C-terminal portion of FAK that can act as a negative regulator of FAK activity. These treatments reduced FAK protein expression and activity and suppressed cell proliferation, motility and invasion[40, 41]. PF-562271, a potent ATP-competitive and reversible inhibitor of FAK, was widely used to block FAK activity. In NSCLC it was shown to increase apoptosis in H125 xenograft model[42].

All these data point to the potential of direct FAK targeting as treatment strategy for fighting invasiveness of NSCLC. Similarly to CXCR4, it was shown that chemo- and radiotherapy could increase FAK activity and tumor cell migration in NSCLC[43-45], which also implies necessity of FAK targeting along with classical therapeutic approaches in this type of cancer. In addition, Lu et al. demonstrated that FAK was associated with resistance to dasatinib in NSCLC cells, and that they could be sensitized by FAK inhibitor, PF-562271, both *in vitro* and *in vivo*[46].

Conclusion

All the available literature data suggest that both CXCR4, as membranous receptor, and FAK, as downstream cytoplasmic tyrosine kinase, are important players in NSCLC pathogenesis. They are primarily involved in regulation of cell's adhesion, migration and invasion and their deregulation in NSCLC lead to increase in metastatic potential of tumor cells. Therefore, it was proved that blocking either CXCR4 or FAK could significantly reduce metastatic processes in NSCLC. Accordingly, these two molecules represent promising targets for treatment of NSCLC in order to prevent and/or suppress its dissemination throughout the body. In addition, preliminary studies have shown that they could be effectively used in individual treatments as well as in combination with conventional therapeutic strategies, particularly in order to overcome the resistance to chemo- or radio-therapy. All the researches

aimed to target CXCR4 and FAK in NSCLC are at the initial stages and require further and more detailed examination and awaiting their final evaluation for possible clinical application in treatment of this type of cancer.

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Discovery of anxiolytic 2-ferrocenyl-1,3-thiazolidin-4-ones exerting $GABA_A$ receptor interaction via the benzodiazepine-binding site

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Herein, the most important data related to the structure of $GABA_A$ receptor and the role of $GABA_A$ agonists–those that interact with the BzD (benzodiazepine) binding site–in the treatment of anxiety are briefly reviewed. This was done in order to give the rationale behind the recently published design and synthesis of a small library of anxiolytic *N*-substituted 2-ferrocenyl-1,3-thiazolidin-4-ones.The starting point in the design was Cook's pharmacophore/receptor model for $GABA_A$ agonists and inverse agonists. *In vivo* and *in silico* (molecular docking) studies suggested that the ferrocene core was the key structural motif that enabled favorable interactions of the title compounds with BzD-binding site. The details regarding the design, synthesis, anxiolytic activity and SAR analysis (structure-activity relationship) will be presented in the oral presentation of this lecture.

Anxiety–a global problem

Encyclopedia of Psychology defines anxiety as an emotion characterized by feelings of tension, worried thoughts and physical changes like increased blood pressure. People with anxiety disorders usually have recurring intrusive thoughts or concerns; they may avoid certain situations out of worry; they may also have physical symptoms such as sweating, trembling, dizziness or a rapid heartbeat.[1]These disorders are the most frequently diagnosed neuropsychiatric diseases in Western countries. According to a recent 3-year multi-method study covering 30 European countries and a population of around 500 million people, anxiety disorders had the highest 12-month prevalence estimates (a total of 14%) compared to all other psychiatric conditions.[2,3] In 1990 costs (either direct or indirect) associated with anxiety disorders–these were estimated to affect 26.9 million individuals in the United States at some point during their lives–were around $45 billion US Dollars, *c.a.* 30% of the

total expenditures for mental illnesses (data refer to US).[3,4] Less than one-quarter of the costs associated with anxiety disorders were for a direct medical treatment; over three-quarters were attributable to lost or reduced productivity.[4,5] It is reasonable to expect that now, 25 years later, a general change in the life style made this problem even more pronounced. Thus, the availability of an effective treatment that could substantially reduce the economic and social burden of these common and often crippling disorders is of the highest importance. Nonetheless, although there has been a growing appreciation of how emotional disorders result from a combination of genetic and environmental risk factors,[6] identifying reliable biochemical biomarkers or genetic variants that can be used to diagnose anxiety disorders and help predict treatment outcomes remains a major challenge.[7]

The role of $GABA_A$ agonists in the treatment of anxiety

In the recent years, an increasing number of anxiety-related preclinical studies was focused on serotonin, neuropeptide, glutamate and endocannabinoid systems; nevertheless, since their discovery in the mid-1950s, drugs targeting the γ-aminobutyric acid A receptor ($GABA_A$ agonists; e.g., the family of benzodiazepine, BzD, compounds) have been and still are the first choice in the treatment of anxiety.[2,8-14] These heterocyclic compounds with fused benzene and diazepine rings (structures of the archetypical benzodiazepines, bromazepam and diazepam, are given in Figure 1) have a variety of therapeutically useful actions, including anxiolysis, sedation, seizure suppression and muscle relaxation and as sedative-hypnoticdrugs they have essentially replaced the barbiturates owing to a substantially improved therapeutic index.[11] These drugs exert their anxiolytic effects by allosterically activating specific $GABA_A$ receptor subtypes to promote inhibitory neurotransmission in the brain.[2]

$GABA_A$ receptors are heteropentameric ligand-gated chloride channels, made up of 19 known subunits: α_{1-6}, β_{1-3}, γ_{1-3}, ρ_{1-3}, δ, ε, θ and π (Figure 1), whose activation typically leads to an influx of chloride.[2,12-17]Many $GABA_A$ receptors contain two α-subunits, two β-subunits and one γ-subunit with two GABA (γ-aminobutyric acid) binding sites formed by α- and β-subunits (Figure 2). Approximately 60% of all $GABA_A$ receptors have an$\alpha_1\beta_2\gamma_2$-subunit combination.[12-14]

Benzodiazepines mediate their action via a modulatory binding site (the benzodiazepine, BzD, site) on most (although not all) $GABA_A$ receptors. The binding site for benzodiazepines is formed by α_1-, α_2-, α_3- or α_5- and a γ-subunit (typically γ_2), which is present in approximately 90% of $GABA_A$ receptors (Figure 2); the α subunit adjacent to the γ_2-subunit determines the sensitivity of the receptor to benzodiazepines. $GABA_A$ receptors containing the α_4- or α_6-subunit do not bind clinically used classical benzodiazepines.[2,12-18] The BzD binding site is located on the extracellular surface of the receptor and it includes amino-acid residues from 6 noncontiguous regions (these are usually designated as Loops A-F) of subunits α and γ. For several classical benzodiazepines even specific amino-acid residues that contribute to the binding are uncovered.[15-18] For example, it is known that the molecule of diazepam

interacts with α1His101, α1Asn102 (Loop A), α1Gly157 (Loop B), α1Val202, α1Ser205, α1Thr206 and α1Val211 (Loop C).[16,17] Histidine to arginine mutations at a conserved residue in the α-subunits functionally abolish the benzodiazepine binding site, while the action of the physiological neurotransmitter GABA is preserved (Figure 2).[12]

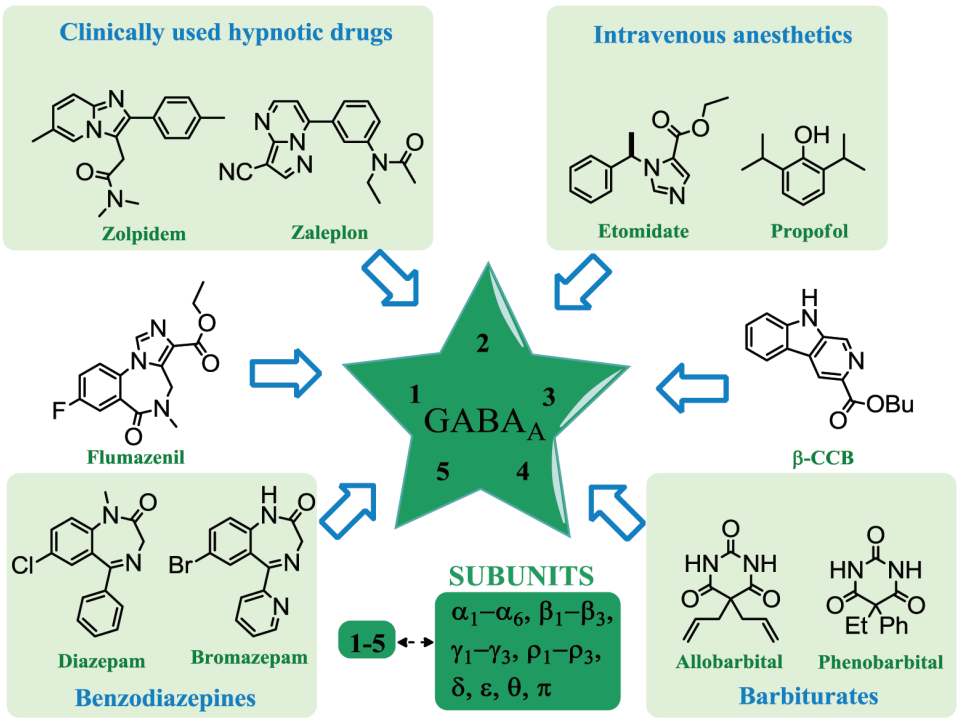


Figure 1. Structural diversity of compounds targeting the heteropentameric $GABA_A$ receptor (there are 19 known types of $GABA_A$ subunits: α_{1-6}, β_{1-3}, γ_{1-3}, ρ_{1-3}, δ, ε, θ and π); diazepam andβ-CCM (methyl β-carboline-3-carboxylate) are prototypic $GABA_A$ positive and negative allosteric modulators, respectively(interaction via BzD-, benzodiazepine-, binding site), while flumazenil is a typical BzD-$GABA_A$ antagonist

Ligands at the BzD binding site are $GABA_A$ allosteric modulators: they modify the efficiency and/or affinity of $GABA_A$ agonists (e.g. GABA), and, thus, regulate their activity. These compounds are sometimes referred as BzD agonists or BzD inverse agonists (it was previously thought that BzD site is an independent receptor).[12-14] $GABA_A$ receptor modulation by benzodiazepine site agonists is self-limiting: the conductance of the channel in the presence of GABA and benzodiazepines is not higher than the conductance that can

be achieved with high concentrations of GABA alone. Moreover, benzodiazepines do not open the chloride channel in the absence of GABA.[12] The structures of prototypic $GABA_A$ (interaction via BzD binding site) positive (diazepam) and negative allosteric modulators (β-CCM; methyl β-carboline-3-carboxylate), as well as its antagonist (flumazenil) are given in Figure 1. In addition to benzodiazepines, the $GABA_A$ receptor is also the major target for the number of other compounds, among which clinically used hypnotic drugs, barbiturates (they bind to the extra membrane domain of $GABA_A$, to the so-called "barbiturate binding site"), some intravenous anesthetics, etc.[12] The structures of some of these compounds are also given in Figure 1.

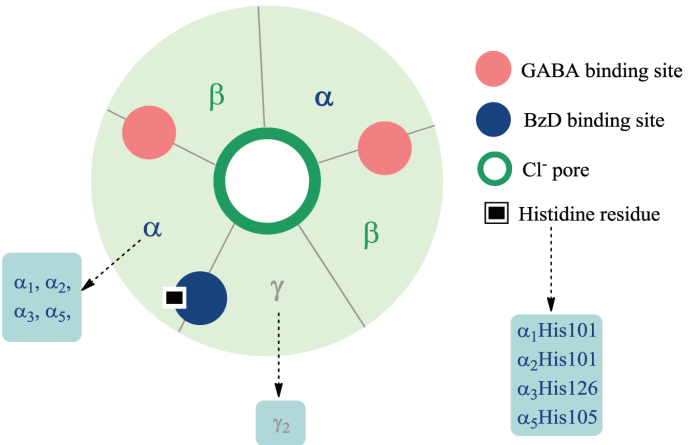


Figure 2. Simplified representation of a typical BzD-sensitive $GABA_A$ receptor (comprised of 2 α- (α_1, α_2, α_3 or α_5), 2 β- and one γ-subunit (usually γ_2)). GABA and benzodiazepine (BzD) binding sites are given as red and blue circles, respectively; ▣ was used to designate His residues (α_1HIS101, α_2HIS101, α_3HIS126, α_5HIS105) from BzD site that are important for the binding of benzodiazepines

Experimental results suggested that the sedative, anterograde amnestic and in part the anticonvulsant actions of diazepam are mediated by α_1-containing $GABA_A$ receptors, that the anxiolytic-like and to a large part the myorelaxant actions are mediated by α_2-containing $GABA_A$ receptors, and that the myorelaxant action is mediated in part by α_3- and α_5-containing $GABA_A$ receptors.[12] Moreover, the development of tolerance to the sedative action of benzodiazepines has been linked to α_5-containing $GABA_A$ receptors, and their addictive properties to α_1-containing $GABA_A$ receptors.[12,19-21] Nonetheless, while experiments with the histidine-to-arginine mutated mouse lines clearly define a role for the mutated $GABA_A$ receptor α subunit if a response to diazepam is absent, they do not formally exclude a contribution of other α-subunits to the response in question.[12,19-21]

The problem with benzodiazepines currently used in the treatment of anxiety is that different pharmacological effects are not clearly separable by dosing. Anxiolytic actions are observed at lower doses than the sedative actions. However, sedation is still a problem when benzodiazepines are used as daytime anxiolytics.[2] In addition, the long-term use of benzodiazepines is hampered by the occurrence of troublesome side effects, including sedation, memory disturbances, tolerance and dependence liability.[2,12] A lot of work has been done on the design and optimization of new anxiolytic agents. Unfortunately, a number of promising candidates were either discontinued due to unexpected side effects or failed clinically owing to toxicity.[12] Having this in mind, it is not surprising that the key challenge of this field ultimately remains in the identification of new medications that are devoid of the limitations in efficacy and tolerability that characterize existing anxiolytics.

Cook's pharmacophore/receptor model

Some 15 years ago, Cook and associates developed pharmacophore/receptor models for BzD-binding sites of three recombinant $GABA_A$ subtypes ($\alpha_1\beta_3\gamma_2$, $\alpha_5\beta_3\gamma_2$, and $\alpha_6\beta_3\gamma_2$), *via* an SAR ligand-mapping approach.[18] This study was based on the affinities of 151 BzR ligands at five distinct ($\alpha_{1\text{-}3,5,6}\beta_3\gamma_2$) recombinant $GABA_A$ receptor subtypes from at least nine different structural families. According to this model potential $GABA_A$ agonists and inverse agonists should be able to interact with the following (sub)sites of the receptor: (i) an H-bond acceptor (A2), (ii) an H-bond donor (H1), (iii) a 'bifunctional' hydrogen-bond donor/acceptor site (H2/A3), (iv) four lipophilic pockets (L1, L2, L3, and LDi), and (v) three sterically forbidden sites (S1, S2, and S3) (Fig. 1a). Hydrogen-bond donor sites H1 and H2, hydrogen bond acceptor A2 and lipophilic region L1 were assigned as four basic anchor points on the receptor protein-complex (Figure 3). The use of Cook's pharmacophore/receptor models for these subtypes has resulted in the design of novel BzR ligands selective for the $\alpha_5\beta_3\gamma_2$ receptor subtype (as previously mentioned, it seems that diazepam acting on α_5-containing $GABA_A$ is not sedative).[12,18]

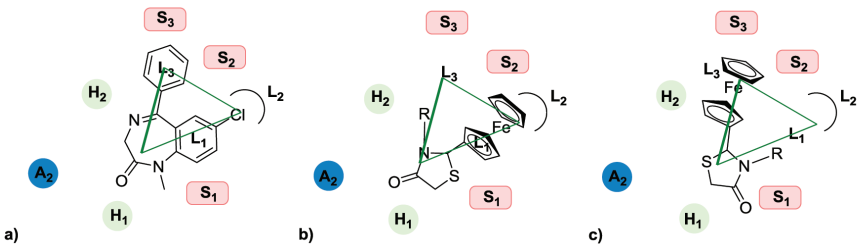
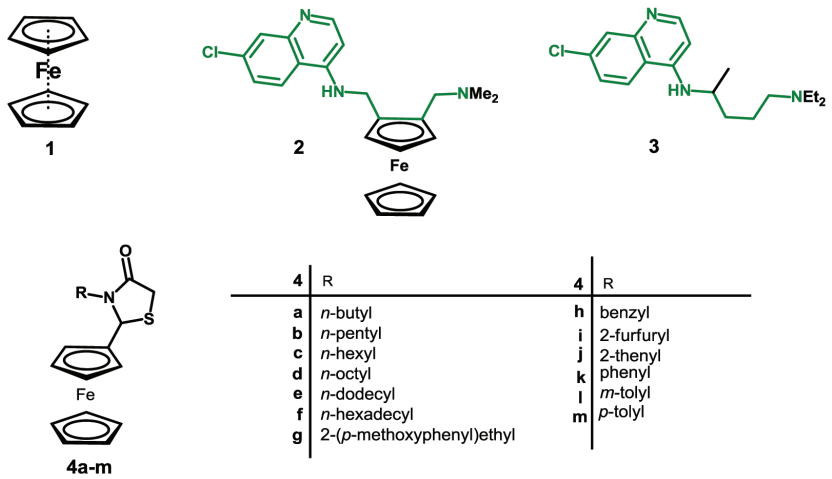


Figure 3. a) Pharmacophore model for the BzD-binding site of the $GABA_A$ receptor (A_1-A_3-H-bond acceptor, H_1, H_2-H-bond donor, S_1-S_3-sterically forbidden sites, L_1-L_3-lipophilic pockets), with diazepam[18] as template; the hypothetical pharmacophore triangle is given in green. b) and c) Fitting of *N*-substituted 2-ferrocenyl-1,3-thiazolidin-4-ones[22] into the proposed model for ligands targeting the $GABA_A$ complex.



4	R	4	R
a	*n*-butyl	h	benzyl
b	*n*-pentyl	i	2-furfuryl
c	*n*-hexyl	j	2-thenyl
d	*n*-octyl	k	phenyl
e	*n*-dodecyl	l	*m*-tolyl
f	*n*-hexadecyl	m	*p*-tolyl
g	2-(*p*-methoxyphenyl)ethyl		

Figure 4. Structures of ferrocene (1), ferroquine (2), chloroquine (3) (common structural motif of 2 and 3 is given in green) and a newly discovered anxiolytic 2-ferrocenyl-1,3-thiazolidin-4-ones (4a-m) exerting $GABA_A$ receptor interaction via the benzodiazepine-binding site.

Figure 3 shows Cook's pharmacophore/receptor model[18] for the BzD-binding site of the $GABA_A$ receptor with diazepam ($GABA_A$ agonist) and *N*-substituted 2-ferrocenyl-1,3-thiazolidin-4-ones (two possible orientations) as templates. The central structural feature of diazepam is the 1*H*-1,4-diazepin-2(3*H*)-one core, which is fused to a substituted (chlorine) benzene ring and bears a phenyl substituent. The hypothetical pharmacophore triangle is depicted in light gray. The Cook's model[18] was the starting point in the recently published design of a small library of 1,3-thiazolidin-4-one derivatives (**4a-m**, Figure 4): the central heteroaromatic core was "expanded" as to fit into the pharmacophoric triangle by the introduction of appropriate substituents to N- and C2-atoms.[22] The logic behind the choice of substituents will be discussed later on and in the oral part of this presentation.

Ferrocene derivatives as potential $GABA_A$ agonists

Ferrocene (**1**, Figure 4) is the prototypical metallocene with a η^5 (pentahapto) sandwich structure; it consist of two cyclopentadienyl rings bound on opposite sides of a central iron ion.[23-29] This compound, accidentally discovered in the middle of the last century, is remarkably stable. Ferrocene and its derivatives are extensively studied and have interesting properties that made them useful as ligand scaffolds, pharmaceutical candidates, reagents and redox standards, precursors to materials and in anti-knock formulations. A number of

studies have shown that the introduction of the ferrocene core (Fc), or a formal exchange of an existing aromatic ring with Fc, may significantly enhance the molecule's (desirable) bioactive properties.[23-29] For example, ferrocene analogues of the antimalarial drug chloroquine (**3**) are active against chloroquine-resistant strains of *Plasmodium falciparum*; one of these, ferroquine (**2**), made it to clinical trials.[30,31] The Fc unit might act as a hydrophobic spacer and/or lipophilicity/bioavailability enhancer (enabling easier passage through cell membranes).[22] It is also known that the ferrocene Fe^{2+}/Fe^{3+} redox chemistry might contribute to the bioactivity of ferrocene derivatives.[32]

As mentioned above, Cook's pharmacophore/receptor model[18](Figure 3) served as a starting point in the design of new BzD agonists. 1,3-Thiazolidin-4-one carbonyl unit is a strong H-bond acceptor. For this reason it was presumed that this compound and its derivatives might favorably interact with H1 receptor (sub)site (Figure 3). In addition, the divalent sulfur atom could either act as a weak H-bond acceptor or eventually may form a favorable S-π interaction with the appropriate arene system at the receptor-binding site.[22]

The critical point in the design of new BzD agonist was an assumption that the ferrocene core, linked to C2 of the heterocyclic ring, would nicely fit L1/L2 (Figure 3b) or L3 (Figure 3c) lipophilic pockets and could possibly act as an "activity enhancer".[18, 23-29] Ferrocene-unit-containing compounds have not been previously exploited as possible $GABA_A$ targeting compounds. Alongside the above mentioned favorable properties of ferrocene, it was assumed that the specific arrangement of two aromatic (Cp) rings in close proximity (*c.a.* 3.5 Å), "positioned" in parallel, would enable additional favorable interactions with the benzodiazepine-binding site.[22]

Identity of the substituent attached to the nitrogen atom (R, Figure 4) was varied in order to gain an insight into possible SAR relationships. Two main types of R substituents were included in the study: aliphatic (*n*-alkyl chains of differing lengths, **4a-f**; "aliphatic analogues") and aromatic (with benzene/furan/thiophene cores, **4g-m**; "aromatic analogues"). Within the "aromatic" series (that was expected to be more active due to possible favorable π-π interactions), the length of the-$(CH_2)_n$- (n=0-2) spacer between the N-atom and the aromatic core was varied. This was done to finely tune the geometric parameters of the structures (a better model fit) as well as toprobe if an increased conformational freedom of the molecule would influence the net activity. Another reason for the synthesis of **4i**, **4j**(heteroaromatics) and **4g** (methoxyphenyl group) was the fact that these compounds contain additional H-acceptors (S/O-atoms) that could increase the ligand's affinity towards the receptor.

The anxiolytic properties of the designed compounds were evaluated in several different *in vivo* models. These experiments confirmed that the designed compounds possess (strong) anxiolytic properties, and revealed2-ferrocenyl-3-(4-methoxyphenylethyl)-1,3-thiazolidin-4-one (**4g**, Figure 4) as the most potent anxiolytic among the library compounds.[22] Involvement of the $GABA_A$ receptor complex in the activity of **4g** was inferred from a set of experiments with known $GABA_A$-targeting agents.[22]

Although the crystal structure of the $GABA_A$ receptor is not yet available, there are several homology models that are in good agreement with experimental findings.[15,16] For example,

Berrgman and co-workers recently developed a unified model of the $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptor complex (the most abundant subunit combination), based on the glutamate-gated chloride ion channel (Figure 5).[15]The designed library compounds **4a-m** (as well as the appropriate standard drugs used in biological assays)were all docked into the extracellular domain (blind docking) of the Berrgman's model of the $GABA_A$ receptor.[22] This was done in order to explore whether the library compounds fit into the BzD-binding site and to possibly rationalize the results of *in vivo* experiments. The results of the docking experiments for the most active derivative, **4g**, are summarized in Figure 5.[22]

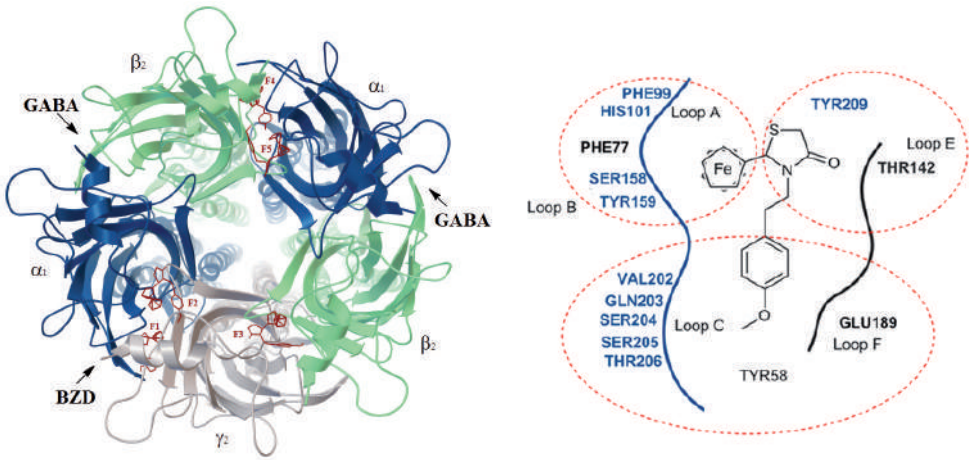


Figure 5. Left: Unified homology model of the $GABA_A$ receptor complex[15] (the shown view is from the side of the receptor's extracellular domain). α_1-subunits are colored blue, β_2 green and γ_2 are light gray; BZD- and GABA-binding sites are marked with arrows; the most favorable docking poses of **4g** are given in red (F1-F5 families of poses).[22] Right: 2D macromolecule-ligand interaction diagram for**4g** docked in the BzD-binding site of $GABA_A$(only amino-acid residues that fall into 4 Å-radius spheres around the ligand are shown); red dashed lines group spatially close entities[22]

Five families of docking poses, F1-F5, were found for this compound (the same is true for other library compounds). Poses belonging to F1 family (at the interface of α_1/γ_2-subunits; these were energetically most favorable ones) placed the molecules into the BzD-binding site. 2D macromolecule-ligand interaction diagrams, which depict the ligand and its (spatially close) interacting residues is also depicted in Figure 5.[22] **4g** was docked into the BzD-binding site in a similar way as known $GABA_A$ agonists/antagonists (e.g. diazepam or flumazenil). The residues of the following 15 amino acids were within the 4 Å sphere (strong interactions expected)[49] around **4g**: α_1HIS101, α_1ASN102 (Loop A); α_1LYS155; α_1VAL202, α_1SER204,

α_1GLY207, α_1GLU208, α_1TYR209, α_1VAL211 (Loop C), γ_2PHE77 (Loop D); γ_2ARG144 (Loop E); γ_2THR193, γ_2ARG194, γ_2SER195 and γ_2ARG197 (Loop F), Figure 12C and 12D. Several residues, namely α_1HIS101, α_1VAL202, α_1SER204, α_1TYR209, α_1VAL211, γ_2PHE77, are known to be important for BzD binding.[24,48] Additional 20 residues were within a 7 Å-sphere (possible interactions) around **4g**: α_1PHE99, α_1GLY103, α_1GLU137, α_1PRO153, α_1SER158, α_1TYR159, α_1GLY200, α_1GLN203, α_1SER205, α_1THR206, α_1VAL210, α_1MET212, α_1THR213, γ_2TYR58, γ_2ASN60, γ_2THR142, γ_2GLU189, γ_2ASP192, γ_2LEU198 and γ_2TRP196. Among these, α_1TYR159 (Loop B), α_1GLY200 (Loop C), α_1SER205 (Loop C) and α_1THR206 (Loop C) are important for the binding of diazepam-type anxiolytics to the $GABA_A$ receptor.[22]

The docking experiments corroborated the assumptions made during the design of **4a**-**m** and justified the introduction of the ferrocene core into the molecules; this metallocene seems to perfectly fit into the BzD-binding site. Alongside the ferrocene core, both *in vivo* and *in silico* experiments confirmed that the introduction of CH_2-spacers between the 1,3-thiazolidin-4-one N-atom and an additional (hetero)aromatic ring was important for their activity.[22] Nonetheless, further work (e.g. an appropriate binding study, experimental LC_{50}) is needed to experimentally confirm the mode of the observed anxiolytic action of these ferrocene derivatives.

Conclusion

To the best of our knowledge, a part of the herein summarized data[22] resulted from a unique study on hybrids of ferrocene and 1,3-thiazolidin-4-one as possible anxiolytic agents. Thus, the mentionedstudy[22] on ferrocene-containing 1,3-thiazolidin-4-ones might be regarded as a commencement of a new chapter in the design of new ferrocene-based anxiolytic sand we believe that many interesting findings are about to follow. Further work on the related ferrocene containing BzD-analogues might result in even better $GABA_A$-targeting compounds. Perhaps some of these future efforts could finally yield the "prefect" anxiolytic agent.

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Cell wall: active component of redox metabolism in plants

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One important function of plant cell walls is to provide protection against (a)biotic stress. However, cell wall is not a passive barrier. It is involved in signaling cascades that are activated in response to stress, and it is prone to dynamic modifications which represent a component of defense and adaptation. Alterations in the redox status of apoplast represent an essential part of both, stress and response. Cell-wall polysaccharides, cellulose, pectin, D-galacto-D-mannan, arabinogalactan, and xylan, exhibit different antioxidative activities against the main reactive oxygen species – hydroxyl radical and superoxide radical anion. In addition, pectin/polygalacturinic acid (PGA) and xylan show a modulating ability to bind redox-active metals - iron and copper. Plants might employ different redox properties of polysaccharides to regulate their redox status by modifying the composition or the chemical properties (of particular components) of cell wall. In this way, plants might respond to acute challenges, but also are capable of developing the resistance to the same stimulus (hormesis) or other stressors (cross-adaptation). PGA converts the highly reactive product of radiation – hydroxyl radical into superoxide which might be further dismutated to hydrogen peroxide (H_2O_2). We propose that PGA/ oligogalacturonic acid/ pectic fragments could represent the initiators of redox signaling cascades in stress response, with H_2O_2 being a downstream secondary messenger, and that PGA might operate as radiation-signaling convertor.

The composition of plant cell wall

Plant cell wall is a three-dimensional insoluble network, composed of many different polysaccharides, including cellulose, hemicelluloses and pectin, proteins and phenolics. This complex and dynamic structure is held together by both covalent and non-covalent bonds. Apoplast, cell wall-including extracellular space outside the plasma membrane, is responsible for the structural integrity of the cell, and is actively involved in growth and responses to abiotic and biotic stress. [1] Primary and secondary cell walls are microfibril-based nanocomposites that differ in the arrangement, mobility and structure of matrix polymers, the higher-order organization of microfibrils into bundles and discrete lamellae, and their roles in the life of the plant.[2] Cellulose is composed of neutral, β-(1-4)-D-glucan

chains, hydrogen-bonded together to form microfibrils. Hemicellulosic polysaccharides are complex molecules that associate with cellulose microfibrils, providing a cross-linked matrix. Hemicelluloses can be divided into four main classes: xyloglucans, which contain a heavily substituted β-1,4-glucan backbone; (gluco)mannans, which contain a variably substituted backbone that includes β -1,4-linked mannose (glucose and mannose) residues; glucuronoarabinoxylans, containing a substituted β -1,4-linked xylan backbone; and mixed linkage glucans, which involve an unsubstituted backbone of glucosyl residues containing both β -1,3- and β -1,4-linkages.[1] Pectins are complex polysaccharides comprised of domains of homogalacturonan (HG), rhamnogalacturonan I and relatively minor amounts of rhamnogalacturonan II. [3,4]

The differing functions of the cell wall are reflected in the large variation and reorganization in cell wall composition between different cell types and during cell differentiation.[2,5] Primary cell walls are synthesized during growth and typically are relatively thin, pliant, highly hydrated structures. Secondary cell walls provide strength and rigidity in plant tissues that have ceased growing. Primary walls are comprised of 15–40% cellulose, 30–50% pectic polysaccharides, 20–30% xyloglucans and low amounts of arabinoxylans and structural proteins, on a dry weight basis, structured into one or more lamellae. According to current depictions of primary walls, cellulose microfibrils are coated by xyloglucans and tethered by them to form a load-bearing network, with pectins functioning as a co-extensive, space-filling matrix that separates the microfibrils.[6] Like primary cell walls, secondary cell walls are composite materials based on 3-nm cellulose microfibrils, with lignin, xylans, and glucomannans replacing xyloglucans and pectins.[7] Secondary cell walls are less hydrated than primary cell walls, containing only about 30% water at saturation. Loosely aggregated bundles of cellulose microfibrils are coated with a disordered xylan–lignin complex. Partially oriented glucomannan chains adhere by hydrogen bonding to the cellulose aggregates and acetylated segments of these glucomannans bridge between the aggregates.[7]

Apoplastic redox system

Many processes in plants catalyze the partial reduction of oxygen resulting in the production of reactive oxygen species (ROS), the most important being: superoxide radical anion ($O_2^{\bullet-}$), hydrogen peroxyde (H_2O_2), and hydroxyl radical ($HO^{\bullet}$).[8,9] In plants, the two major sites of ROS production are the photosynthetic and respiratory electron transport chains. In the light, the main source of ROS are chloroplasts and peroxisomes, while in the dark, mitochondria are the main ROS generators. ROS are detected in almost all intracellular organelles, as well as at the plasma membrane and in the apoplast. Pertinent to this, apoplast contains a number of enzymes that are involved in ROS metabolism, such as NADPH oxidases, peroxidases and several types of oxidases, including oxalate oxidases and amine oxidases.[10,11,12] Plant NADPH oxidases are encoded by respiratory burst oxidase homologue (Rboh) genes that are found in a broad range of plant species.[13,14] They have been shown to play key roles in numerous physiological processes and various stress

responses.[15] Rbohs contain six conserved transmembrane helices, and cytosolic flavin adenine dinucleotide (FAD)- and reduced nicotinamide adenine dinucleotide phosphate (NADPH)-binding domains in their C-terminal regions, as well as two N-terminal Ca^{2+}-binding (EF-hand) motifs.[16] NADPH acts as cytosolic electron donor to apoplastic O_2, which is reduced to $O_2^{\bullet-}$ via FAD and two independent heme groups. ROS produced by NADPH oxidases are involved in cell growth, development, and responses to (a)biotic stressors.[14,17] Class III secretory plant peroxidases (EC 1.11.1.7; donor: hydrogen peroxide oxidoreductase) are mainly cell wall- and vacuole-located, heme-containing enzymes that catalyze the one-electron oxidation of several substrates by using H_2O_2 as an oxidant. Peroxidases can function in both ROS-consuming and ROS-generating reactions.[18] ROS produced by peroxidases are suggested to be important in various developmental processes, such as cell wall loosening and cross-linking, as well as in several stress types (*e.g.* pathogen-induced defense reactions).[19,20] The oxidative function of peroxidases requires the presence of a reductant in the apoplast. Several compounds, such as NAD(P)H, ascorbate, thiols, phenols and fatty acids have been shown to act as reducing agents under *in vitro* settings.[21] However, the nature of the native apoplastic reductant(s) is not known yet. Copper-containing amine oxidases (CuAOs, E.C. 1.4.3.6) and polyamine oxidases (PAOs, EC 1.5.3.3) are abundant in plant cell walls and catalyze the oxidative deamination of di- and polyamines with H_2O_2 as a common product.[22,23,24] CuAOs are homodimers with a copper ion and a 2,4,5-trihydroxyphenylalanine quinone cofactor present in each subunit, whereas PAOs are flavin-containing, monomeric enzymes.[23,24] PAOs regulate the cellular levels of di- and polyamines, which are ubiquitous, polycationic compounds essential for cell growth and development.[23,25] H_2O_2 produced in the apoplastic polyamine catabolism is used in cell wall modifications during plant growth and in the response to biotic and abiotic stresses. The germin protein family is comprised of two main subgroups in plants, oxalate oxidases (OXOs) and germin-like proteins (GLPs).[26] Germins are oligomeric, apoplastic proteins that possess oxalate oxidase activity, whereas GLPs do not usually have this activity.[26,27] Oxalate oxidases contain Mn in structure and catalyze the oxidation of oxalate to CO_2 and H_2O_2.). These proteins are implicated in a variety of plant processes including germination, development, pollen formation, and response to abiotic and biotic stresses.

Non-enzymatic response to stress on the level of cell wall is most likely based on the release of transition metals, such as iron and copper. The concentration of Fe in the leaf apoplastic fluid is between 5 and 30 μM. Most of the iron (more then 95%) in the leaf apoplast is bound to cell wall components.[28] The pool of redox-active iron can be increased in response to cell wall damage. The increase in the level of 'free' iron (and copper) can dramatically change redox settings in the apoplast by interacting with ascorbate or phenolics that are present in the apoplast.[29] It has been estimated that the total concentration of ascorbate (ascorbate + dehydroascorbate) in apoplastic fluid is in the 0.5–2 mM range.[30,31] These interactions involve molecular oxygen,[32] and have H_2O_2 and $HO^{\bullet}$ as final products (Figure 1).

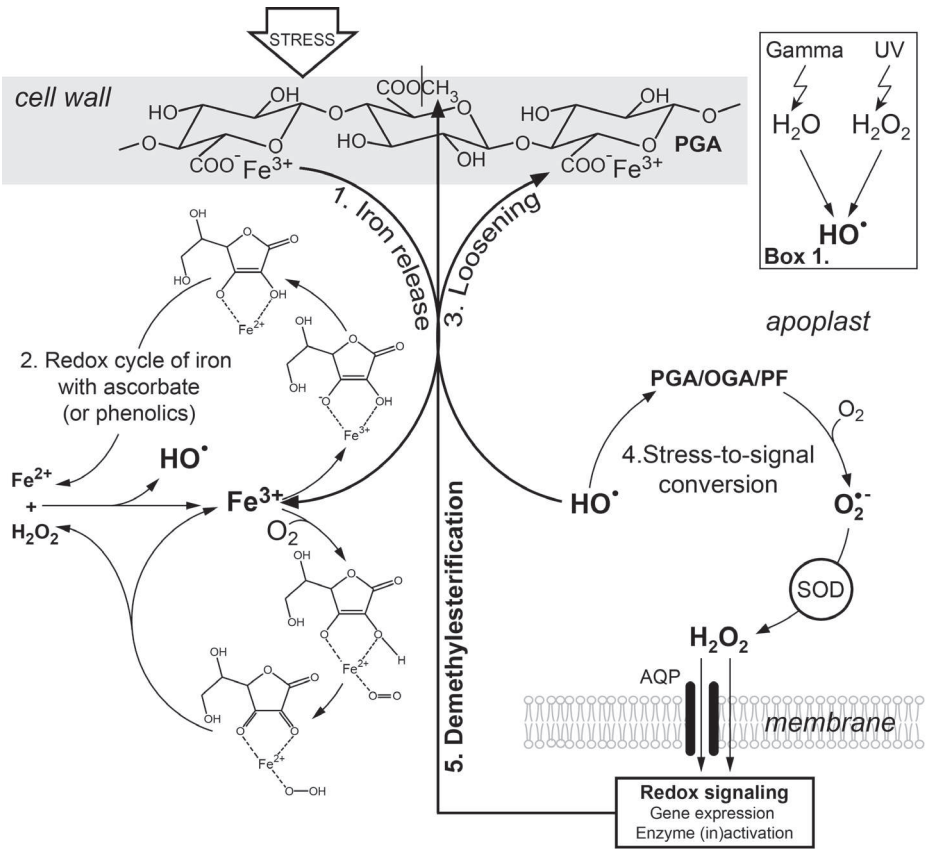

Figure 1. Non-enzymatic redox system and response to stress in the apoplast. In response to stress, Fe^{3+} (that is mainly bound to polygalacturonic acid (PGA)) is released from the cell wall (1). In the apoplastic fluid, iron is involved in redox cycles with ascorbate (phenolics) and O_2 (2), which have hydroxyl radical ($HO^{\bullet}$) as a product. $HO^{\bullet}$ can further loosen cell wall to promote further Fe^{3+} release (3), or it can be converted by PGA/oligogalacturonic acid (OGA)/pectic fragments (PF) to superoxide ($O_2^{\bullet-}$) which is dismutated by superoxide dismutases (SOD) to H_2O_2 (4). H_2O_2 can enter the intracellular compartment either via specific aquaporins (AQP) or via lipid bilayer, to activate signaling cascades and gene expression. The response can be silenced by H_2O_2-activated demethylesterification (*i.e.* methyl esterase activation), which expose $-COO^-$ groups thus increasing the capacity of cell wall to bind Fe^{3+}. Box 1: Irradiation-induced $HO^{\bullet}$ production.

The highly reactive $HO^{\bullet}$ radical is generated via Fenton (-like) systems:[33]

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + HO^{\bullet}$ (1)
$Cu^{2}+ + H_2O_2 \rightarrow H^+ + Cu^+H_2O^{\bullet}$ (2)
$Cu^+H_2O^{\bullet} + H_2O_2 \rightarrow Cu^+ + HO^{\bullet} + O_2 + H_2O$ (3)
$Cu^+ + H_2O_2 \rightarrow Cu^{2+} + HO^{\bullet} + OH^-$ (4)

In addition $HO^{\bullet}$ can be produced from H_2O_2 by UV irradiation (homolysis) or via gamma-irradiation provoked hydrolysis.

This is only a part of a well-developed set of feedback loops. Hydroxyl radical is known to break cell-wall polymers and to provoke wall loosening, which might have further release of metals as a result. Since iron forms coordination bonds at least partially with carboxyl groups, this degradation leads to redistribution of iron pool from the wall into the apoplast. Of note, according to Pearsons's HASB principle Fe^{3+} is a hard acid whereas Fe^{2+} is a borderline acid. Since carboxyl group is generally hard base, it preferentially binds Fe^{3+}, and shows lower affinity for Fe^{2+}.[34] Hence, redox-active Fe^{2+} in the apoplast is not inhibited by the cell-wall components. PGA/OGA/PF have an important role in the maintenance of these redox loops (explained in the next chapter). CuZn-superoxide dismutase (CuZn-SOD) has been found in the apoplastic fluid[35,36] and the cell wall.[37] In addition, the presence of Mn-SOD has been demonstrated in the cell wall of moss and in the apoplast of conifera.[38,39] All these data speak in favor of the production of $O_2^{\bullet-}$ and H_2O_2 in the apoplast. The final feedback - demethylesterification appears to be in charge of quieting down pro-oxidative activity of iron. It has been shown that H_2O_2 upregulates several cell wall-relevant enzymes, including pectinesterase.[40] Demethylesterification of pectin increases the number of free carboxyl groups and, consequently, the capacity of the cell wall to bind iron. On the other hand, demethylesterification of pectin also promotes the transformation capacity for $HO^{\bullet} \rightarrow O_2^{\bullet-}$ (oxidative burst- signaling) conversion.

Because ROS can be toxic if present in excess, whereas some (H_2O_2 and $O_2^{\bullet-}$) at physiological concentrations participate in signaling cascades,[41] plants require at least two different mechanisms to regulate ROS concentrations: one that enables the detoxification of excess of ROS developing under pathophysiological conditions, and one, that is in charge of the fine modulation of low levels of ROS inherent to signaling cascades. For the latter, plants prevalently rely on ascorbate, which is considered as the most abundant low-molecular-weight antioxidant in the apoplast.[41] Ascorbate acts as both enzymatically-regulated redox buffer and redox-sensing metabolite.[42] However, excessive ROS production, which may overwhelm ascorbate-based antioxidative system, develops in plants exposed to biotic and abiotic stress.[43]

Antioxidative activities of cell wall polysaccharides

It has been proposed that the changes in the molecular size and quantities of cell-wall polysaccharides might be involved in the response to various environmental factors related to

oxidative stress,[44,45] as well as in growth control.[46] For example, it is known that the accumulation of metal-binding polysaccharides in the cell wall represents an important element of defense strategy against heavy metal toxicity.[47] In addition, cell-wall polysaccharides are subjected to many modifications, which may vary among different cell types, during cell differentiation and development and in different seasons and under different environmental conditions.[2] For example, HG in pectin can be methyl esterified and acetylated to a different extent.[4] Such pectin modifications appear to be initiated in response to pathogens.[48]

Our recent studies have demonstrated the contribution of major cell-wall polysaccharides: cellulose, pectin and hemicelluloses (D-galacto-D-mannan, arabinogalactan, and xylan) to the redox buffering capacity of the apoplast. Cell-wall constituents showed different effects on $HO^{\bullet}$ radical production via iron and copper-mediated Fenton reaction.[49] The rank order of antioxidative activity against $HO^{\bullet}$ production in the iron-involving Fenton system was: xylan = pectin > D-galacto-D-mannan > arabinogalactan > cellulose. The rank order of antioxidative activity in $HO^{\bullet}$-generating system in the Fenton-like system with copper was: xylan = pectin > arabinogalactan = D-galacto-D-mannan = cellulose. The production of additional reactive species was observed in two systems: $O_2^{\bullet-}$ in the presence of pectin and carbon dioxide radical ($CO_2^{\bullet-}$) in the presence of xylan. It appears that xylan and pectin represent the main carriers of activity against $HO^{\bullet}$ production via Fenton system in the cell wall. There are three different modes of acting against $HO^{\bullet}$ radical production in the Fenton system: (i) the scavenging of $HO^{\bullet}$; (ii) metal chelation, which inhibits the reaction; (iii) binding of the metal in such a manner that iron participates in the Fenton reaction, but that the close proximity of the compound (polysaccharide) increases the probability of $HO^{\bullet}$ scavenging near the site of production. It should be stressed that $HO^{\bullet}$ almost unselectively reacts with any biomolecule nearby, which results in a very short half-life time of $HO^{\bullet}$ in biological systems of approximately 10^{-9} s.[50] The most efficient inhibitors of $HO^{\bullet}$ production, xylan and pectin, contain carboxyl groups. It has been reported that compounds containing two or more specific functional groups such as –COOH, –OH and others, in a favorable structure–function configuration can show metal-binding activity.[51] At natural apoplast pH, carboxyl groups in the cell wall are deprotonated, thus carrying negative charges.[52] Hence, it seems plausible that xylan and pectin show high inhibitive effects against $HO^{\bullet}$ production due to their ability to bind metals ions. Relatively rigid structure and wide spatial distribution of $–COO^-$ groups do not allow bending of polysaccharide chain and complete chelation of metal ions. So it seems that the third mode of 'anti-Fenton' activity is predominant for pectin and xylan. This 'scenario' is also the most desirable by living systems, having the scavenging of notoriously reactive $HO^{\bullet}$ radical and a safe degradation of H_2O_2 as results. It is important to note that according to reactions 2–4, copper can remove H_2O_2 without the involvement of a reductant. On the other hand, iron requires a reducing agent (e.g. phenolics) to be converted from redox inert Fe^{3+} to active Fe^{2+} form within cell wall. Iron deficiency induced secretion of phenolics facilitates the reutilization of root apoplastic iron in red clover.[53] Such antioxidative activity might be particularly important for buffering oxidative burst in plant's extracellular compartment, taking into account that: (i) under settings in the

apoplast ascorbic acid exists in anionic form (ascorbate) and is repulsed by negative charges in the cell wall, probably making ascorbate incapable of covering entire extracellular space with antioxidative protection; and (ii) ascorbate cannot directly scavenge H_2O_2 to prevent its leakage to surrounding healthy tissue.

The antioxidative activity against $HO^{\bullet}$ observed for other cell wall polysaccharides, cellulose, arabinogalactan, and D-galacto-D-mannan, might be attributed to the formation of coordination bonds with metals via –OH groups.[51,54] Such bonds are weaker than ionic bonds created between metal ions and $–COO^-$,[55] probably accounting for the lower antioxidative activity observed. Our study revealed some other details that might upgrade the knowledge about structure–antioxidative activity relationships among polysaccharides. Cellulose showed higher antioxidative activity in a copper involving reaction in comparison to the iron-based Fenton system, while D-galacto-D-mannan showed just the opposite effects. Coordination chemistry of Fe^{2+} and Cu^{2+} differ, where Fe^{2+} most frequently shows a coordination number of six, while Cu^{2+} principally forms four coordination bonds.[55] According to the results from our study, cellulose polymer is organized in such a manner to promote the formation of coordination bonds with copper, while D-galacto-D-mannan preferentially binds iron. Pertinent to this, it has been documented that the cell wall shows different affinities for various divalent cations.[56] A very low antioxidative activity of cellulose might also be attributed to its rigidity and insolubility.

Another interesting fact is that the exposure of pectin and xylan to $HO^{\bullet}$ led to the production of additional reactive species, $O_2^{\bullet-}$ and $CO_2^{\bullet-}$, respectively. Carbon dioxide radical can be converted to carbon dioxide. For example, it has been proposed that $CO_2^{\bullet-}$ can be converted to CO_2 by SODs.[57] In relation to this, the reaction of xylan with $HO^{\bullet}$ might at least partially account for CO_2 emission from plants exposed to oxidative stress.[58] In addition, $CO_2^{\bullet-}$ appears to target metallocenters of different proteins and might provoke their inhibition.[57] It is noteworthy that we have found that the methylation of carboxyl groups on oligogalacturonic acid (OGA) alters the production of secondary reactive products in the Fenton system. Namely, methylated OGA produced carbon-centered radical instead of $O_2^{\bullet-}$ (unpublished data). In contrast to different antioxidative activities against the Fenton system, the cell-wall polysaccharides examined showed relatively similar activity against $O_2^{\bullet-}$.[49] This implies that they act by the blunt scavenging of superoxide, not showing specific interactions. Distinctive antioxidative properties of different cell-wall polysaccharides imply that plants could modify the composition of the cell wall in order to 'tune' redox conditions in the apoplast and to complement antioxidative protection provided by Asc.

Cell wall polygalacturonic acid as a source of superoxide in the apoplast

During the last few years, novel insights into the mechanisms translating combinations of different stimuli or stresses into distinct response patterns have illustrated how a plant can perceive different stimuli simultaneously, and integrate the resulting signals to generate

quantitative, adaptive responses.[59] In the apoplastic space different types of sensors exist that can perceive stimuli originating in the environment or deriving from the plant itself.[60] The stimuli can either have a chemical (ligands, fragments) or a physical character (exemplified by plasma membrane distortion or displacement of membrane *vs.* wall). Examples for plant-derived chemical stimuli are cell wall fragments, metabolites or peptides while invading pathogens represent sources of non-plant-derived stimuli.[61] These two types of chemical stimuli can initiate specific responses, thus allowing detection of invading pathogens and initiation of specific defense responses.[62] Physical stimuli on the other hand can be indicative of abiotic stress (reductions in turgor pressure due to drought stress) or changes in the mechanical characteristics of cell walls. [63]

UV-B radiation has been reported to affect plant biochemistry, physiology and gene expression.[64,65] This may result in altered biomass allocation, timing of plant development and senescence, branching, leaf and canopy architecture, chloroplast structure and nucleus integrity.[66,67] UV-B radiation affects the pathways of secondary metabolites and the accumulation of phenolic compounds.[68] It induces the impairment in photosynthetic function and an increase in the antioxidative capacity.[69,70,71] At least some of these changes are related to the interplay between UV irradiation and the metabolism of ROS.[68] When exposed to UV, plants show increased production of ROS which might initiate DNA, protein and membrane damage.[72, 73,74,75] On the other hand, emerging data imply that UV may activate various signaling pathways.[76,77] Several lines of evidence indicate that stress response to UV significantly overlaps with plant response to some other ROS-involving events, such as mechanical damage, pathogen attacks or the exposure to ozone.[72, 78,79] Further, ionizing radiation, such as X and gamma, is recognized to create perturbations in the homeostatic equilibrium as well as reversible or irreversible damage. However, some low dose radiation effects in plants are not in accordance with the linear no-threshold dose response model. It has been reported that low dose gamma radiation provokes in plants various physiological changes on the cellular level: promoted expression of specific genes, e.g., those for antioxidative defense enzymes, hormesis, cross-adaptation (to UV-B irradiation, high-intensity light, and drought stress), increased biomass yield, plant vigor and growth, and grain yield, fruit ripening, enhancements of embryogenesis, and additional trichome formation (reviewed in [80]).

Pectin is structurally and functionally the most complex polysaccharide in plant cell walls. Multiple lines of evidence indicate a role for pectin in plant growth, development, morphogenesis, defense, cell–cell adhesion, wall structure, signaling, cell expansion, wall porosity, binding of ions, growth factors and enzymes, pollen tube growth, seed hydration, leaf abscission, and fruit development.[81] HG is the most abundant pectin polysaccharide, making up to 65%of total pectin.[82] The structural domains of pectin are built on more or less methyl- and acetylesterified galacturonan. One main characteristic of pectin is the extent of methyl-esterification on the carboxyl group of PGA. The degree of HG methyl-esterification has been reported as the key determinant of plant and organ development involving processes

such as cell division, expansion, and adhesion.[83] Furthermore, a minimum stretch of nine unmethylated galacturonic acid residues can form Ca^{2+} linkages, which may promote the formation of so-called ''egg-box'' model structure. Hence, the methyl-esterification status of HG can have dramatic consequences on cell wall texture and mechanical properties, thereby contributing to cell shape and growth.[83] PGA/OGA/pectic fragments (PF) represent apoplastic signaling molecules involved in defense regulation.[83]

In the recently published papers[84,85], we have shown that the components of cell wall – galacturonic acid polymers (PGA, OGA, and PF) are capable of transforming $HO^{\bullet}$ into $O_2^{\bullet-}$ (Figure 1). The mechanism involves the reaction of $HO^{\bullet}$ with carboxyl groups in galacturonic acid moieties, which results in the production of carbon-centered radicals - carbon dioxide radical and pectin C(5) radical.[84,85] These radicals further react with molecular oxygen having $O_2^{\bullet-}$ as an end product. The step that is enabled by PGA is huge. Namely, PGA 'takes' the most reactive species in the living systems - ·OH which has exclusively damaging effects, and turns it into $O_2^{\bullet-}$, which is approximately three orders of magnitude less reactive and might act as a signaling molecule.[41,86] In our experimental setups, three $HO^{\bullet}$-generating systems were used: Fenton reaction, Haber-Weiss-like reaction and UV-provoked homolysis of H_2O_2. We propose that PGA/OGA/PF could represent the initiators of redox signaling cascades in stress response, with H_2O_2 being a downstream secondary messenger. We examined the redox effects of UV irradiation on cell wall isolates from *Pisum sativum* leaves, and PGA and galacturonic acid, in the presence of H_2O_2.[84] Our results shed a new light on: (1) the redox-modulating role of cell wall; (2) the production of superoxide in the extracellular compartment; (3) the mechanisms involved in translating UV stress into molecular signaling and (4) some other UV-related phenomena in plants, such as CO_2 emission.

Our results clearly add up to the understanding of the mechanisms by which UV irradiation sets off constructive changes, but the implications go far beyond, as some other types of environmental radiation, such as gamma, possess enough energy to produce $HO^{\bullet}$ from water. In this case, signaling cascades may be also initiated by H_2O_2 which is produced from $O_2^{\bullet-}$ by SOD. Pertinent to this, the increased level of H_2O_2 in plants exposed to gamma radiation is well documented, the highest concentrations being observed in leaves,[87] while SOD activity has been closely related to the adaptive responses provoked by low dose radiation.[88,89] Hydrogen peroxide is the key species in redox signaling showing the ability to modulate different biological processes by activating/inhibiting gene transcription and enzyme activity.[41] Even more, at concentrations in the higher physiological range, H_2O_2 induces more permanent, modifying changes, such as hormesis and cross-adaptation.[41] In close, we propose that polygalacturonic acid may operate as radiation-signaling convertor. Medium-to-high dose gamma irradiation has been shown to induce drastic inhibition of PGA-decomposing enzyme polygalacturonase and the upregulation of pectin methylesterase,[90] thus preserving PGA structure and at the same time making carboxyl groups on pectin available for conducting $HO^{\bullet} \rightarrow O_2^{\bullet-}$ conversion. This most likely has a role in antioxidative defense (promoted $HO^{\bullet}$ removal and iron binding), maintenance of cell integrity and activation of adaptive mechanisms.

Conclusion

Antioxidative activities of cell wall polysaccharides and pectin capacity to transform $HO^{\bullet}$ into $O_2^{\bullet-}$ suggest a dynamic redox-modulating role of plant cell wall components.

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A perfect match - Combinatorial effect of Estrogen, Raloxifen and Methotrexate on the induction of apoptosis in human endometrial cell line

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Uterine myomas are the most common gynecologic tumor in women of reproductive age. These tumors develop as a result of locally high estrogen in uterus, absence of progesterone component and systematically low level of blood circulating estrogen. Symptomatology of myoma uteri is presented in the forms of abnormal uterine bleeding, infertility during reproductive period and also a possibility of malignant alteration. Treatment options of the uterine myomas consist of surgical, medical and interventional therapy, such as uterine artery embolization. Using cell line, derived from a patient with myoma, in our experiment we have investigated effects of combinatorial application of substances. Our aim was to both investigate and determine what combination of investigated drugs caused lowering of the cell number. Our results demonstrate that a perfect match of investigated substances induced apoptosis in ThESC cell line.

Introduction

Uterine myomas are the most common gynecologic tumor in women of reproductive age. These benign tumors develop as a result of locally high estrogen in uterus, absence of progesterone component and systematically low level of blood circulating estrogen. As a result of this hormone disbalance, numerous changes occur. First of all, as a result of low circulating estrogen, changes in the bone tissue arise in the form of osteoporosis; secondly, the high level of estrogen in uterus combined with the absence of progesterone component causes the uncontrolled proliferation of the uterine cells. This represents a crucial event in the development of benign tumors. Symptomatology of myoma uteri is presented in the forms of abnormal uterine bleeding, infertility during reproductive period and also a possibility of malignant alteration.

In order to express its abundant effect on different cells and organs, the obligatory step for the estrogen action implies bounding of estrogen to estrogen receptors (ER), both alpha and beta. Different expression of ER in various tissues determines multiple diverse effects of estrogen and it results in the activation of the ligand-dependent estrogen signaling. As a

result of the estrogen-receptor interaction, cell activates the transcription of the co-regulatory proteins and promotion of the transcription of the estrogen responsive genes [1]. Due to its ability to modulate transcription of the genes, crucial either for the cell survival or cell death, estrogen represents the powerful hormone whose levels orchestrate the fine border between health and disease development. The primary physiological effect of estrogen is portrayed in the cell proliferation, differentiation, activation of vasculogenesis and induction of higher mitotic cell index, consequently leading to the higher level of incidence for transcriptional mistakes resulting in the tissue tumor genesis [2].

Treatment of breast cancer cell line with high concentration of estrogen causes higher proliferation rate compared to the proliferation rate in case of the lower dose estrogen treatment. Deprivation of estrogen induces apoptotic changes in MCF-7:5C cells [3]. Although the molecular mechanisms of estrogen-induced apoptosis are not fully understood, some evidence indicates that mitochondria-related caspase pathways are involved [4].

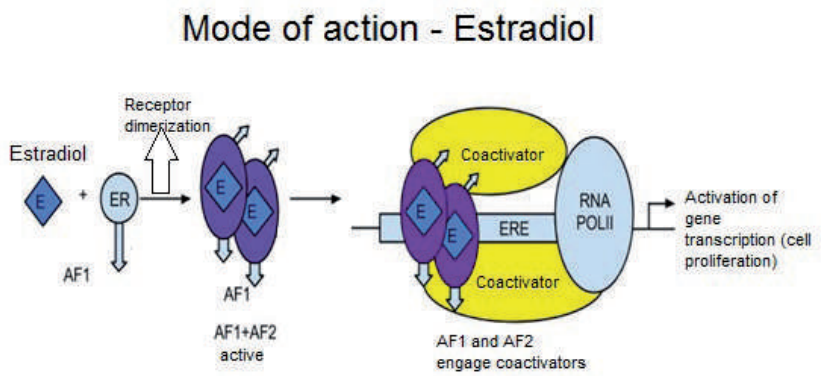

Figure 1. Mechanism of action- Estradiol.

Treatment options of the uterine myomas consist of surgical, medical and interventional therapy, such as uterine artery embolization. One of the medicaments that was investigated for the treatment of proliferative changes in uterus caused by estrogen is Raloxifene, SERM (selective estrogen receptor modulator). Raloxifene is the only selective estrogen receptor modulator approved for the long-term treatment in the prevention of osteoporotic fractures and for the reduction of invasive breast cancer risk in post-menopausal women. Despite the fact that Raloxifene was registrated for the treatment of osteoporotic changes, recent studies showed its multiverse positive effects on bone metabolism, lipid metabolism, coagulation pattern, menopausal symptoms, breast cancer onset and endometrial cancer onset. These

positive effects arise as a result of Raloxifene modulatory effects on estrogen receptors in different tissues. While use of another SERM, Tamoxifen, increases the risk of endometrial hyperplasia and malignancy, Raloxifene has neutral effects on the uterus. Raloxifene binds to both ER α and β with high affinity. However, the binding affinity to ERα is four times higher compared to its binding to ERβ [5]. In bone and liver tissue, Raloxifene demonstrates purely agonistic effect [6], while its antagonistic effect in the form of inhibition of proliferation and induction of apoptosis is present in various cancer cell lines [7, 8]. Moreover, the SERMs are used in the treatment of endometrial hyperplasia [9], however there are no experimental data showing the cytotoxic and apoptotic effects of Raloxifene on human endometrial stromal ThESC cell line isolated from the patient's myoma uteri.

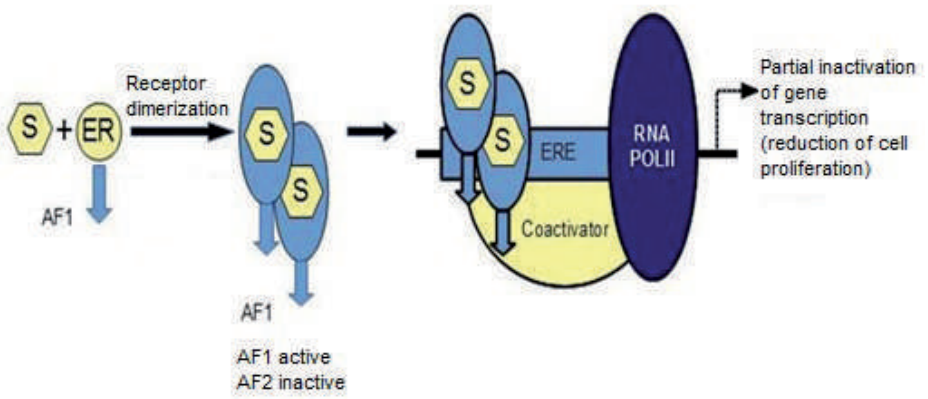

Figure 2. SERM mechanism of action.

Apoptosis is crucial for the tissue homeostasis, embryogenesis and its deregulation leads to the autoimmune disorders, immunodeficiency, or cancer. It could be induced by various stimuli activating either extrinsic or intrinsic apoptotic pathway [10]. Anti-apoptotic family of Bcl-2 proteins serves as a marker of the survival mechanism of the cell. David et al. [11], showed that the high expression of anti-apoptotic Bcl-2 proteins preserves the integrity of the mitochondrial outer membrane, inhibits translocation of pro-apoptotic protein Bax from the cytosol to the outer membrane of mitochondria, blocks the releasing of cytochrome c from intermembrane space of mitochondria into the cytosol, utterly inhibiting induction of apoptosis [11, 12]. Activation and translocation of Bax to mitochondria could be considered as a marker for the mitochondrial apoptotic pathway [11]. Following the initiation of the inner apoptotic pathway, cytochrome c with APAF-1β forms functional apoptosome that activates pro-caspase 9. Active caspase 9 directly activates executor pro-caspases 3 and 7 that finalize the apoptotic process [11].

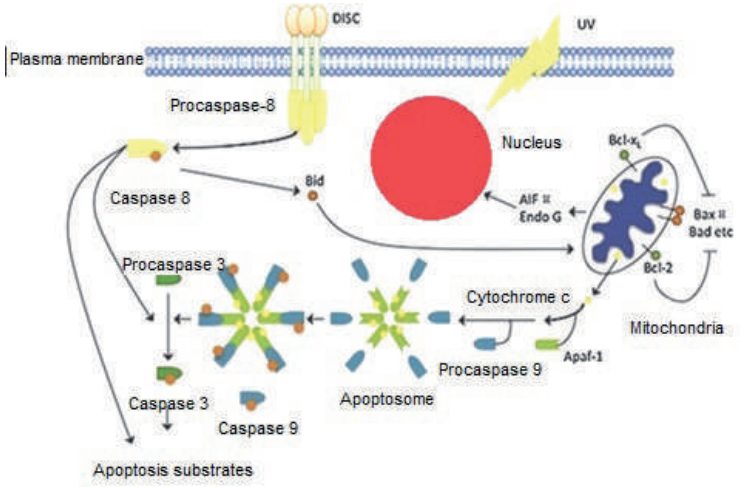

Figure 3. Induction of apoptosis.

Although numerous studies demonstrated an apoptotic effect of Raloxifene on certain cell lines [19, 20] and patients [20], the exact apoptotic mechanism of Raloxifene is not yet fully understood. Liu et al. [20] suggested that low doses of Raloxifene inhibit the growth of cultured leiomyoma cells due to down regulation of the expression of anti-apoptotic Bcl-2 [20], however its exact apoptotic mechanism has not been determined. In same study, along with the increasing concentrations of Raloxifene, the effect of estrogen on the cell proliferation was also determined suggesting the positive effect of estrogen on cell proliferation.

Methotrexate formerly known as amethopterin, is an antimetabolite and antifolate drug. In clinical study conducted by Zivanovic et al., [14, 15] authors showed that local application of low dose of Methotrexate (MTX) reduced both the volume of myomas and menstrual bleedings consequently improving haematological status of patients [14]. The use of MTX reduced incidence of invasive methods of the treatment. Methotrexate is used for the treatment of lymphomas, osteosarcomas, breast, ovarian, lung and urinary bladder cancers [16-25] rheumatoid arthritis, psoriasis [14, 15]. Methotrexate competatively inhibits dihydrofolate reductase (DHFR) thus preventing involment of folic acid in the de novo synthesis of nucleic acids and proteins [26]. Savion et al., [27] showed that MTX is directly involvement with BAX regulation, a Bcl-2 family group molecule [27], key regulators of the apoptotic processes [28].

Our aim was to find the right and the most efficient combination of different types of drugs that could be used as a potential treatment of these benign changes. In order to investigate and determine efficiency of different doses of investigated substances, in our experiment we have evaluated both cytotoxic and apoptotic effects of different single and combined doses of estrogen, Raloxifene and Methotrexate on ThESC cells line.

Cytotoxicity

In this study, the cytotoxic effect of different doses of Raloxifene (from 10^{-5} M to 10^{-10} M), Methotrexate (16, 8, 4 and 1μM) and estrogen (10^{-9}, 10^{-8}, 10^{-7} and 10^{-4} M) were investigated using (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) MTT assay. Different types of treatment with investigated substances on human endometrial cells lasted for 24 hours. The aim of this experiment was to determine single dose of investigated substances that caused the lowest cytotoxic effects. These smallest single doses of drugs were then combined in pre, co and post-treatment in order to investigate potential additive or synergetic effect in their combined cytotoxic effect (in this study we have only presented the results for the lowest doses of investigated drugs). After 24 hour period , cytotoxic effects of investigated substances were clearly distinguished and did not exceed 10% of cytotoxic cells. For the purpose of our experiment and according to MTT assay, doses that induced lowest cytotoxic effects for Raloxifene were 10^{-9} M (5.09%), Methotrexate 1 μM (9.29%) and estrogen 10^{-4} M (8.59%) , respectively. Once we have determined doses of interest, in our next MTT assay, we investigated effects of various types of drug application (combination of drugs) and compared their cytotoxic effects to the cytotoxic effects of their single drug application. Our results demonstrate interest findings. In the case of pre, co and post-treatment with Raloxifene and estrogen, one combination of investigated drugs gave significant cytotoxic effect. This treatment was in the case of simultaneous application of Raloxifene and estrogen. The cytotoxic effect of this combination was statistically significantly higher (22,56%), compared to the cytotoxic effects of single administrations of investigated substances (Raloxifene 10-9 M - 5,09% and estrogen 10-4 M – 8,59%) (Figure 4) and in all other types of the treatments (results not showed).

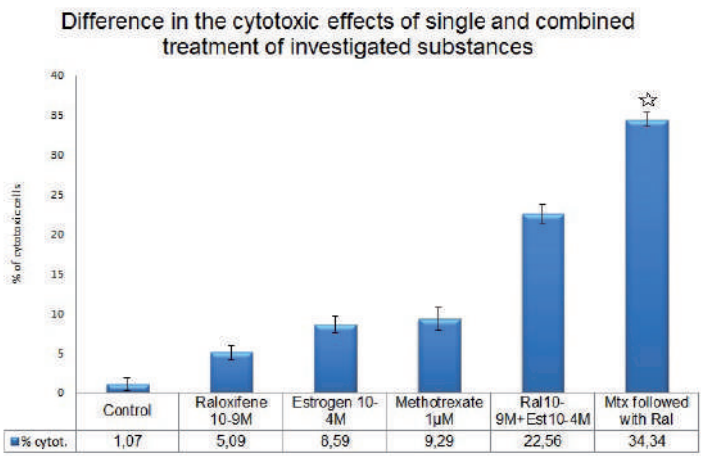

Figure 4. Both single and combined cytotoxic effects of Raloxifene and estrogen in human endometrial stromal cell line.

Moreover, based on the results of MTT assay, we demonstrated that the highest percentage of cytotoxic cells was presented in the case of treatment of the combination of Methotrexate pretreatment followed with Raloxifene.

Following the determination of the cytotoxic effects, next we have investigated the percentage of apoptotic cells after the treatment with investigated drugs using ANNEXIN V/ FITC staining.

In accordance to the results obtained by MTT assay, our results of ANNEXIN V FITC staining showed similar results for all the investigated drugs. The percentage of the apoptotic cells in the case of single drugs administration was – for Raloxifene at 10-9 M (5,75%), estrogen at 10-4 M (8,61%) and Methotrexate at 1μM (9,03%), respectively. However, the significant increase in the percentage of the apoptotic cells and the strength of apoptotic changes were present in the case of the combined drugs application. The most efficient combination and the inducer of apoptotic changes in human endometrial cell line was the combination of Methotrexate pretreatment followed by Raloxifene treatment expressing 35,87% of apoptotic cells. Other combination (Raloxifene 10-9 M and estrogen 10-4 M) showed the lower apoptotic changes compared to the previous combination, but still this combinations was statistically significant in the induction of apoptotic changes (Ral+estr –23,01%) compared to the apoptotic effects of the single drugs administration (Raloxifene 10-9 M – 5,75%, estrogen 10-4 M – 8,61%) (Figure 5).

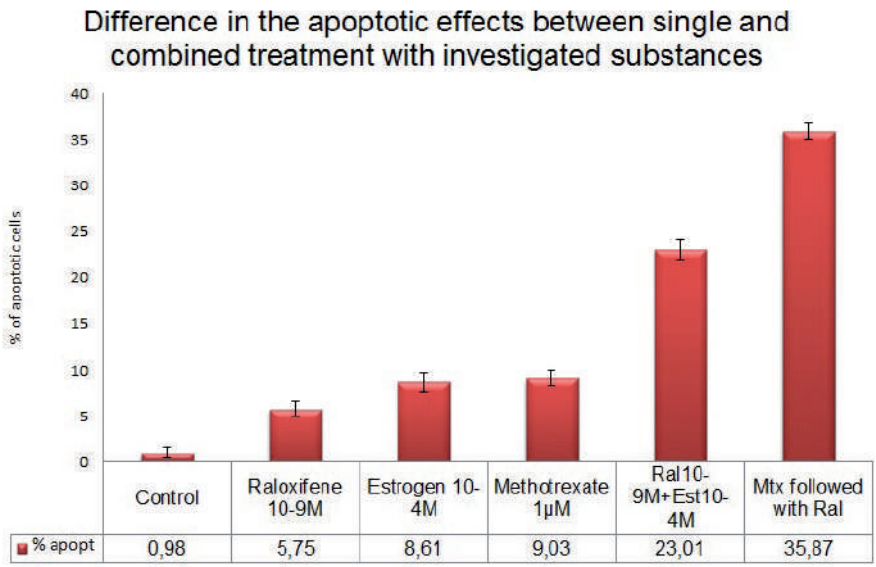

Figure 5. Difference in the percentage of apoptotic cells after treatment with investigated substances compared to the control cell

Fluorescence microscopic analysis of cell death

Following our determination of both cytotoxic and apoptotic effects of the investigated substances, next we have analyzed the expression and the activation of the crucial proteins involved in the programed cell death, apoptosis. First, we have determined the expression of the anti-apoptotic Bcl-2 protein in treated cells and compared it to the control cells. Anti-apoptotic Bcl-2 is associated with prolonged cell survival through inhibition of a common pathway of cell death. One of the first indicators of ongoing apoptotic changes in treated cells is down regulation of anti-apoptotic Bcl-2 protein expression. As a result of reduced Bcl-2 expression, survival mechanisms of treated cells are diminished resulting in their death. Immunofluorescence assay revealed the highest level of Bcl-2 expression of 96% in untreated cells. Single application of Raloxifene 10-9 M (61,41%) and estrogen 10-4 M (48,13%) decreased the Bcl-2 protein expression respectively compared to untreated cells (Figure 6). However expression of Bcl-2 protein was lowest in the case of Methotrexate and Raloxifene combination (15, 99%) compared to control group and to all other drug applications. Reduction of Bcl-2 expression in treated cells suggests diminished survival mechanism that ultimately leads to apoptosis.

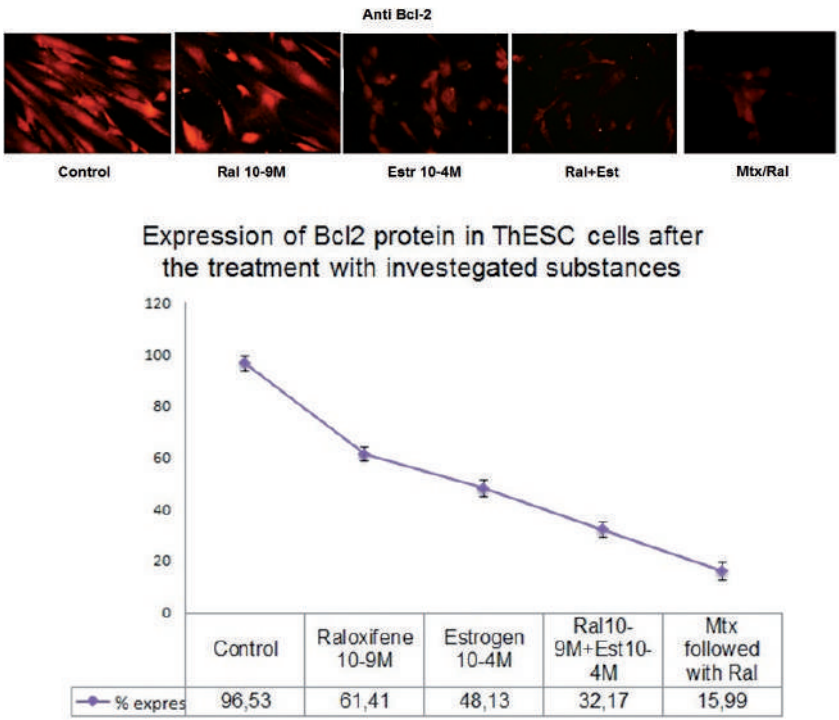

Figure 6. Expression of Bcl-2 protein in the control group of the cells and in cells treated with investigated substances

Pro-apoptotic Bax is mainly localized in the cytosol of the healthy mammalian cells and upon induction of apoptotic signaling, it translocates from cytosol to the mitochondria where it forms transition pores and allows release of cytochrome c that further commits cells to death. When Bax is activated, it undergoes conformational changes that expose its N terminus, and this alteration can be detected by the conformation-specific antibodies. To investigate the level of expression of activated Bax, we performed immunofluoroscence assay using an antibody specific to the N terminus of Bax in treated and control cells (Figure 7). Results indicated that the highest expression of Bax was present in the case of combinatorial treatment of Methotrexate and Raloxifene (57,12%). This expression of Bax is statistically significantly higher compared to the its expression in cells treated with single drug application (Raloxifene 10-9 M- 19,97%, estrogen 10-4 M- 26,77%) as well as in the case of Ral+estr combination (32,11%), respectively. Bax was found to be more activated in cells treated with investigated drugs compared to the untreated cells. Activation of Bax in control cells was most probably due to the spontaneous apoptosis in these cells.

In our next experiment, we investigated the expression of the main apoptotic executioner, caspase 3. Immunofluorescence was performed with an antibody specific to the cleaved active form of caspase-3 following different drug treatment in ThESC cells. Our results demonstrated significantly higher expression (activation) of caspase 3 in ThESC cells treated with investigated substances (Figure 8). However, the combined drug administration (Raloxifene + estrogen) showed statistically higher expression of active caspase 3 (35,05%) compared to the expression that was found in the case of single drug administration Raloxifene 10-9 M (15,42%), estrogen 10-4 M (22,38%) and control cells (1,91%). Results for the expression of active caspase 3 in the case of Methotrexate administration are not presented

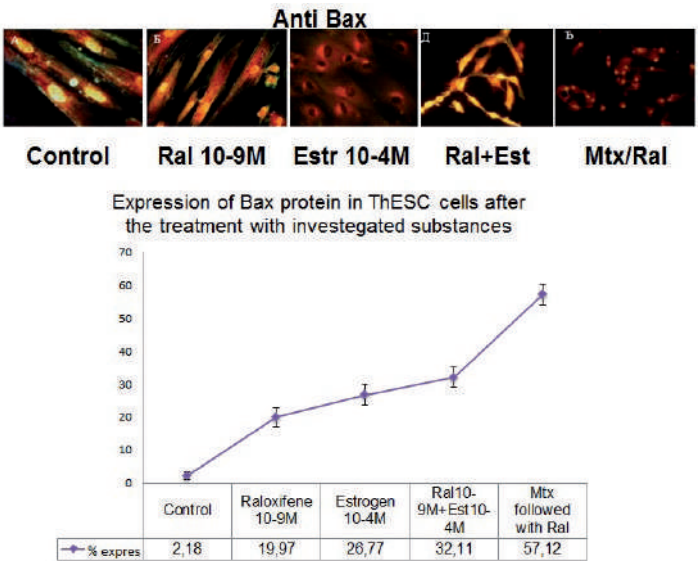

Figure 7. Expression and localization of pro-apoptotic active Bax (N – terminal Bax) in treated cells compared to the control

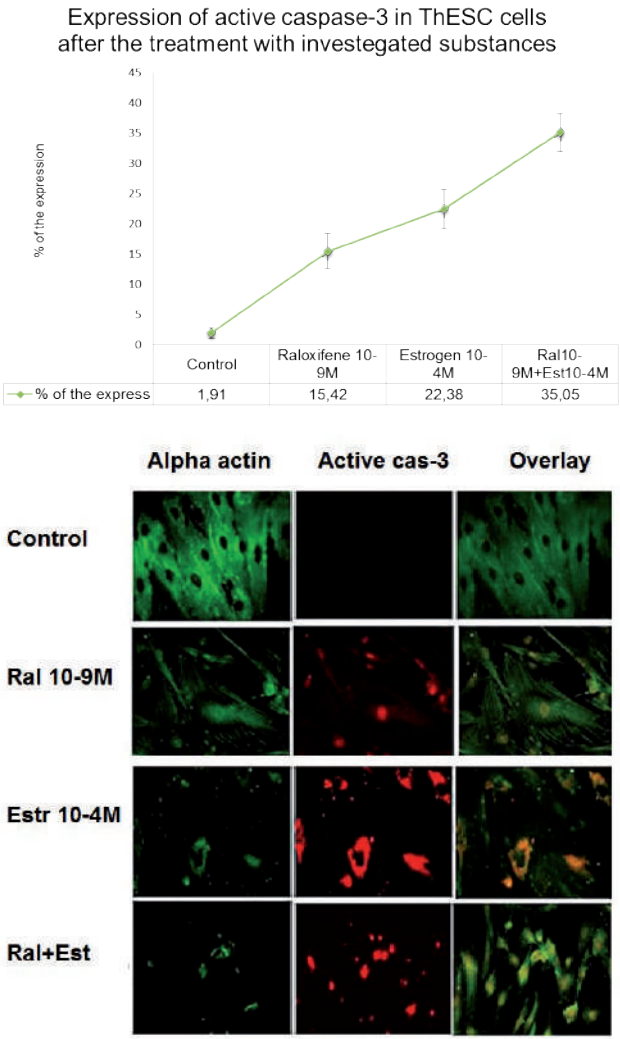

Figure 8. Expression of active caspase 3 in treated cells

Conclusion

Based on the results of our experiments it could be suggested that the combined application of Raloxifene and methotrexate, as well as the combination of raloxifene and estrogen are more efficientXpotential therapies for the treatment of diseases caused as a result of hormonal (estrogen) disbalance compared to the therapeutic effect of the single dose treatment of the same drugs.

Acknowledgements

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Malignant risk stratification of thyroid fine needle aspiration specimens using serum, genetic and protein markers

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Introduction

Papillary thyroid cancer (PTC) is the most common type of malignant thyroid tumor constituting more than 80% of thyroid malignancies.[1,2] Even though PTC is a well differentiated cancer generally with a favorable prognosis, it may become invasive and develop regional metastases in lymph nodes, and even distant metastases.[3-5] Thus, the identification of reliable markers of thyroid malignancy that would facilitate differential diagnostics or improve prognostic predictability of thyroid tumors is of great importance.

Fine needle aspiration (FNA) has been employed in examination of thyroid nodules for many years, and cytology of the aspirates is the primary determinant for whether thyroidectomy is indicated. Although FNA has its shortcomings, such as that inadequate sampling may lead to PTC misdiagnosis, it serves as a good initial diagnostic procedure to exclude clinically significant PTC before performing more invasive measures such as thyroidectomy.

We aimed to improve preoperative diagnostics by introducing serum, genetic and protein markers. FNA biopsy samples from 210 patients were analyzed using general patient information (age, gender, type of thyroid surgery performed), hormonal status (TSH, T3, T4, FT3, FT4, PTH), thyroglobulin and calcitonin serum levels, autoimmune antibody levels (Tg-Ab, TPO-Ab and TSHR-Ab), genetic markers (BRAF V600E, RET/PTC1, RET/PTC3) and caveolin-1 as a protein marker.

Our goal was to determine which parameters are significantly different in benign vs. malignant patients, as well as whether introducing protein and genetic markers improves

cancer diagnosis. In addition, we set out to determine a possible link between Hashimoto's thyroiditis (HT), an autoimmune inflammatory disorder, and PTC, i.e. if HT predisposes patients to develop PTC.

Each patient had blood drawn before FNA biopsy, for the thyroid hormone and autoantibodies measurement. Final histopathological diagnosis of thyroid cancer and HT diagnosis were obtained by the pathologist.

Results

Immunocytochemical staining of FNA samples for caveolin-1 confirmed our previous findings that patients diagnosed with malignancy have an increased caveolin-1 expression.[6] In malignant samples around 60% had a high caveolin-1 expression, while in benign samples, we detected high expression in only 25%. Additionally, BRAF coincided with malignancy, with only one mutation found in benign lesions. When T3 serum levels were abnormal, we observed a trend of increased T3 levels in benign and decreased T3 levels in the malignant patients. Our analysis also demonstrated that compared to standard cytology of FNA, the introduction of molecular and serum markers leads to an increase in diagnostic accuracy.

Next, we analyzed the cancer incidence in PTC patients with and without Hashimoto's thyroiditis, resulting in 37.5% and 43.8% respectively. This implies that there is no correlation of HT with PTC and cancer risk is not elevated in HT patients compared to patients without Hashimoto (risk ratio 0.856, p value 0.557). Having in mind the low sample size (number of patients with HT was 27), we were not able to make a definitive conclusion about HT and PTC correlation.

Conclusions

There is a statistically significant upregulation of caveolin-1 expression in malignant FNA samples compared to benign.

BRAF V600E mutation together with caveolin-1 and the T3 serum levels should be evaluated for cancer diagnosis in FNA biopsies.

Molecular analysis using genetic and protein markers combined with cytology and serum markers may contribute to standard FNA cytology.

In our setting, Hashimoto's thyroiditis was not a risk factor for papillary thyroid cancer.

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Cucurbitacin E inhibits effector cytokine production in encephalitogenic cells

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Introduction

Cucurbitacin E (CucE) belongs to triterpene class of biochemical compounds and can be found in plants of Cucurbitaceae family. It has been shown that CucE possess anti-tumor, anti-oxidative and anti-inflammatory properties. Experimental autoimmune encephalomyelitis (EAE) is an animal model of multiple sclerosis, a chronic inflammatory disease of the central nervous system. IFN-γ-producing Th1 and IL-17-producing Th17 are considered as the major encephalitogenic cells in multiple sclerosis and EAE. Effects of CucE on production of IFN-γ and IL-17 in encephalitogenic T cells were investigated in this study.

Materials and Methods: Dark Agouti rats were immunized with myelin basic protein (MBP) emulsified in complete Freund's adjuvant to evoke active EAE. Draining lymph node cells (DLNCs) were isolated from the rats on the 6th day after the immunization. Different concentrations of CucE were tested on DLNCs re-stimulated with MBP for 24 hours. The production of IFN-γ and IL-17 was measured by ELISA. MTT test was performed in order to assess cell viability.

Results

CucE significantly decreased production of IFN-γ and IL-17 in MBP-stimulated DLNCs. The observed decrease was dose-dependent. Reduction in cell viability was significant in the highest doses applied, but it was minor in comparison to the reduction in the cytokines production.

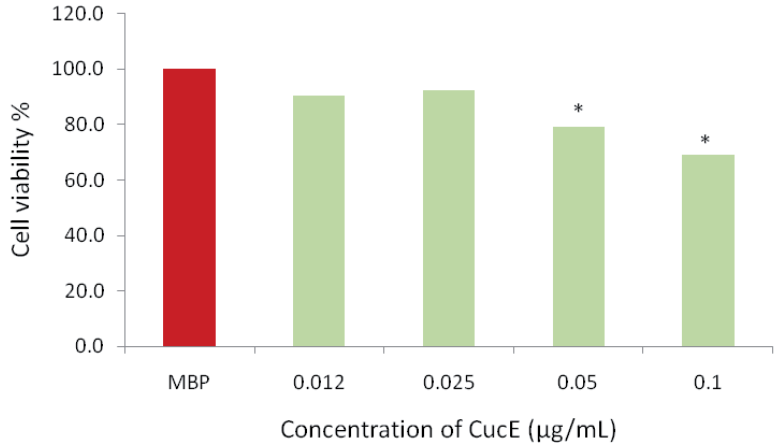

Figure 1. Cell viability of DLNCs treated with different concentrations of CucE.
Three rats per group. *p<0.05 statistical significance to MBP only.

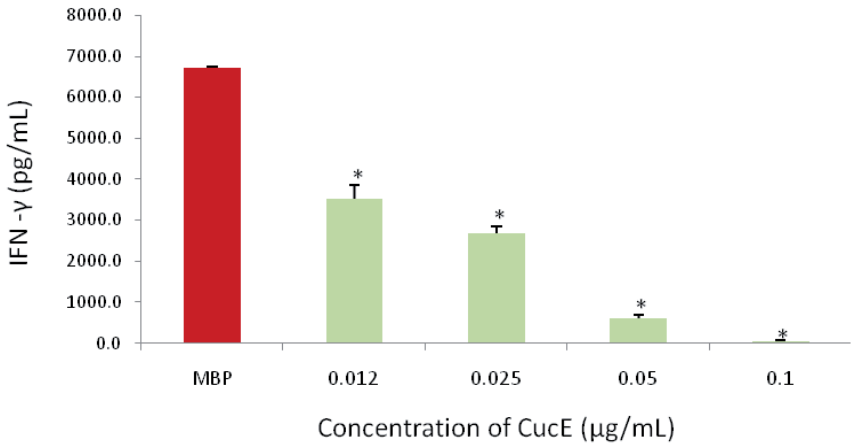

Figure 2. . IFN-γ production in DLNCs treated with different concentrations of CucE.
Three rats per group. *p<0.05 statistical significance to MBP only.

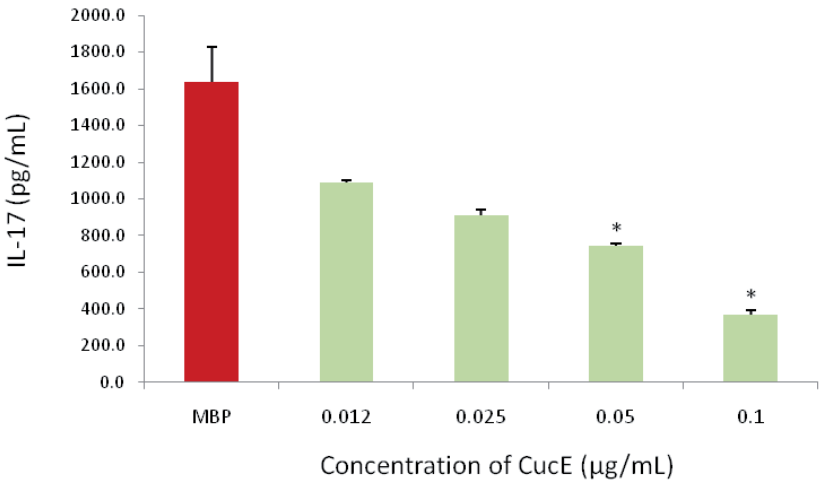

Figure 3. IL-17 production in DLNCs treated with different concentrations of CucE. Three rats per group. *p<0.05 statistical significance to MBP only.

Conclusions

These results clearly show anti-encephalitogenic potency of the CucE. Further research on the potential therapeutic effects of CucE in EAE is warranted.

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Semi rational design of glucose oxidase from *Aspergillus niger* for oxidative stability

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Abstract: Glucose oxidase (GOx) stands out as important industrial enzyme, used in food preservation, textile bleaching, glucose biosensors and enzymatic biofuel cells. It catalyzes oxidation of β-D-glucose producing δ-gluconolactone and hydrogen-peroxide. While presence of hydrogen peroxide is important in food preservation and textile bleaching, it has harmful effects in biosensors and biofuel cells. It has been shown that glucose oxidase loses activity during prolonged use, and it was suggested that reason for that was oxidative damage caused by hydrogen peroxide. One of the amino acid residues that are susceptible to oxidation is methionine. In this study we examined eleven methionine residues present in glucose oxidase from *Aspergillus niger* as potential spots for protein re-design. Two main criteria were used for identification and classification of methionine residues: distance from active site and solvent accessibility. Based on these criteria seven residues were selected and saturation mutant library was made for each methionine residue. Assay for glucose oxidase activity produces hydrogen-peroxide, therefore use of soluble protein for oxidative stability measurement wasn't possible. Therefore, we have used yeast surface display technique, where enzyme is immobilized on cell surface and have developed appropriate oxidative assay using hydrogen-peroxide. Made libraries were first measured for glucose oxidase activity, and only mutants showing more than 30% of wild-type GOx activity were screened for oxidative stability. Oxidative stability was measured in 50mM hydrogen-peroxide for 1 h in the presence of 100mM glucose. Mutants showing higher oxidative stability and at least 60% of wild-type GOx activity were sequenced. After analyzing sequencing results, we decided to combine three methionine positions with each other and test combined mutants for oxidative stability. Surprisingly, triple mutant didn't show any activity. Of double mutants, highest oxidative stability showed one combined mutant, but its stability was comparable to stability of single mutants. For detailed analysis of best enzyme mutants they will be re-cloned, expressed extracellularly and purified.

Calcium-activated chloride channels and Na_2S-induced relaxation of non-pregnant rat uteri in estrus

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The uterus is a spontaneously active tissue, whose contractions have to be controlled and regulated for successful pregnancy and parturition. Spontaneous contractions might be initiated by spontaneous pacemaker activity, although pacemaker cells are not fully defined. Myometrial membrane potential changes are essential for uterine activity which is a function of ion movement across the membrane governed by ion channel activity [1]. Changes in membrane potential are achieved by coordinated actions of two classes of channels: those that exert hyperpolarizing current and those that exert depolarizing currents. Calcium-activated chloride channels (CaCCs) are important contributors to depolarizing currents [1,2]. However, the molecular identity of CaCCs is still not known. Several candidates have been proposed including bestrophins (BEST), CLCA and the anoctamin (ANO or TMEM) family of proteins [3].

Improper or irregular uterine activity may underlie the common pathological disorders such as infertility, improper implantation, dysmenorrhea, weak uterine contraction during labor and preterm labor. The contractile activity of the uterus is regulated by the complex electrophysiologic network which is highly sensitive to various pharmacological and signaling molecules.

Hydrogen sulphide (H_2S) appears to be an important signaling molecule in rat uterus. The production of H_2S and the presence of enzymes responsible for its endogenous production (cystathionine beta-synthase and cystathionine gamma-lyase) have been demonstrated in rat uterus [3,4]. Hydrogen sulphide reduces uterine contractility, and it is recognized as a promising treatment for uterine disorders. It decreases amplitude as well as frequency of uterine contractions. H_2S effect on the frequency of contractions appears to be mediated via pacemaker channels. CaCCs channel inhibitors were shown to reduce the frequency of spontaneous contractions in myometrial strips and were proposed to be the main pacemaker channels in smooth muscles [1]. Very little, however, is known about these channels in the myometrium.

The mechanism of the H_2S -induced relaxation in non-pregnant uteri has not been examined. Organ bath studies were employed to assess the pharmacological effects of sodium sulphide (Na_2S; hydrogen sulphide donor) in uterine strips by exposing them to Na_2S with or without Cl^- channel blockers (DIDS, NFA, T16Ainh-A01, TA). Relaxation was not affected by majority of CaCC modulators since T16Ainh-AO1, tannic acid and NFA failed to inhibit

Na_2S induced relaxation but is DIDS sensitive [3]. DIDS was recently found to be highly selective for bestrophin (BEST-1) channels [5].

BEST proteins were shown to recapitulate the properties of native CaCCs. Although both bestrophin and calcium-activated chloride channel families were proposed to be the candidate genes for smooth muscle contraction their exact function and regulation remain to be confirmed [6].

The aim of this study was to explore the expression of bestrophins channels (BEST-1 and BEST-2) in rat uterus in estrus. Expression studies of the BEST-1 and BEST-2 were performed by Western blotting, RT-PCR and immunohistochemistry. BEST-1 and BEST-2 are expresses at the mRNA level and at protein level in rat uterus in estrus, suggesting a role for BESTs in the control of uterine contractility. However, expression of BEST-1 is higher comparing to BEST-2. Moreover, BEST-1 seems to be major mediators of Na_2S induced uterine relaxation. Mechanistic insights of possible Na_2S-induced modulation od BEST-1 were performed by molecular docking studies.

Taken together, work undertaken strengthens the evidence of a physiologically important role for bestrophin channels in the normal physiology of uterine contractions. Moreover, is an important modulator of uterine contractions and bestrophins appear to be main modulator of its effects. This research add to our understanding of molecular mechanisms of H_2S effects and will be beneficial in designing future in vivo studies, and ultimately identifying new therapeutic targets to treat uterine disorders that are associated with disturbed contractility.

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Isolation, characterisation and the role of complexes formed between transferrin and insulin-like growth factor-binding protein 3

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The insulin-like growth factor (IGF) system plays an important role in the regulation of cell growth, development and differentiation, in both physiological and pathophysiological conditions. Beside two peptide hormones IGF-I and IGF-II, this system includes the following regulatory elements: six IGF-binding proteins (IGFBP-1 to -6), specific receptors for IGF (IGF-1R and IGF-2R), insulin receptor (IR), hybrid receptor (IGF-R/IR) as well as IGFBP specific proteases. When IGFBPs are proteolytically cleaved, IGF-I/II are able to exert their physiological function by interacting with specific receptors on the cell surface.

Approximately 70-75% of the total IGFs are transported in the circulation in the form of ternary complexes with IGFBP-3. Besides being the principal carrier of IGF molecules, IGFBP-3 was reported to exert a number of activities which are IGF-independent. Due to its structural sub-domain organisation, this molecule is able to interact with binding partners other than IGFs, and consequently activate mechanisms and signaling cascades with independent or even opposite effects to those of IGF-I/II, such as apoptosis. One of these binding partners is transferrin (Tf), the principal iron transporter in the blood.

Tf is a molecule of a great importance for the metabolism of iron, an essential ion for physiological processes in the organism. Tf transports iron ion through the circulation, all the way to the place of its action, where it is being internalized after the interaction of Tf with its specific receptor (transferrin receptor, TfR). Being the main carrier protein for iron, Tf reflects it's availability in different physiological conditions (exercise, pregnancy, anemia, liver diseases, and various cancers).

Although the existence of IGFBP-3/Tf complexes has already been reported, the results presented in this PhD thesis offer the first complete structural and functional characterisation of the complexes, together with the analysis of their potential role. The fully optimised method for the isolation of intact IGFBP-3/Tf complexes from serum and tissue samples has been described [1], some structural characteristics have been defined, the effect of iron and other factors on the formation of complexes was studied, their concentration in sera from healthy persons and patients with impaired iron metabolism was measured, as well as their subcellular distribution in colon tissue (healthy and tumor tissue).

The formation and the concentration of IGFBP-3/Tf complexes in persons with anemia, persons with very high iron concentration or patients with colorectal carcinoma (CRC) have also been investigated. The emphasis was made on samples from patients with CRC, a disease accompanied by systemic anemia and increased iron accumulation in cancer cells. CRC was a model system for the analysis of IGFBP-3 internalisation via Tf-TfR pathway.

The concentration of IGFBP-3/Tf complexes in healthy adults was measured to be 241 ± 62 μg/L, which makes up to 5-7% of the total IGFBP-3. The results have shown that in impaired iron metabolism, the formation of complexes is highly dependent on the iron concentration, then IGFBP-3 concentration, as well as the concentration of other proteins involved in iron metabolism, such as ferritin [2].

IGFBP-3/Tf complexes isolated form serum obtained from CRC patients, demonstrated altered glycosylation pattern, increased protein oxidation and a higher affinity for metal ions [3]. Experiments with tissue samples revealed high co-localisation of IGFBP-3/Tf/TfR, especially on the membranes of cancer cells, confirming that the internalisation of IGFBP-3 in CRC pathology is predominantly mediated by the Tf-TfR interaction [3]. The increased presence of TfR on the surface of cancer cells compensates for the reduced IGFBP-3 in the circulation, thus enabling IGFBP-3 internalisation and the expression of its pro-apoptotic role.

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Lysine acetylation of major Chlamydia trachomatis serovar B antigens

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Abstract

Chlamydia trachomatis (Ct) occular servoar B is a common human pathogen causing trachoma – the most common cause of blindness. Several prevention strategies have been proposed, but the vaccine strategy would be the most reliable and cost effective to achieve the greatest impact. Lysine acetylation could affect infectious potential and pathogenicity of Ct. Although post-translational modification such as lysine acetylation occurs in prokaryotes, this is the first study that investigates PTMs of chlamydial proteins.

We prepared three different Ct preparations and purified infectious particles (Ct elementary bodies). Purified samples were analyzed by SDS PAGE-based shotgun proteomics followed by nanoLC-MS runs on LTQ Orbitrap XL. We investigated the acetylation pattern of seven important chlamydial proteins: MOMP, 60 kDa chaperonin, EF-G, enolase, Pmp B, E and F. 60 kDa chaperonin, EF-G, and Pmp B showed the highest degree of acetylation out of the inspected proteins.

Our data suggest that important Ct antigens could be post-translationaly modified by acetylation of lysine residues at multiple places. For a better understanding of trachoma pathology and selection of suitable antigen candidate for trachoma vaccine development, total acetylome of Ct should be further investigated by MS-based strategies that also involve enrichment of modified proteins by immunoprecipitation.

Keywords: *Chlamydia trachomatis*, lysine acetylation, antigens, mass spectrometry

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Development of high resolution 2D map of *Musa acuminata* proteins using combinatorial peptide ligand libraries for the detection of novel allergens

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Background: Food allergy has been recognized as an important health problem in Europe affecting about 3% of adults and about 6% of children [1]. Beside the classical food allergies to milk, eggs, peanut, soy, fish, and crustaceans, allergic reactions can be associated with the consumption of fresh fruits, especially banana, kiwi, apple or peach.

Molecular basis of banana (*Musa acuminata*) allergy has been related to five allergens: profilin (denoted as Mus a 1), class I chitinase (denoted as Mus a 2), non-specific lipid transfer protein (LTP, denoted as Mus a 3), thaumatin-like protein (TLP, denoted as Mus a 4), and β-1,3-glucanase (denoted as Mus a 5) (http://www.allergen.org/). However, there are much more IgE reactive proteins from this allergen source left to be identified and characterized [2].

Development of high resolution two dimensional protein maps by electrophoretic techniques (i.e. 2D PAGE) has become an important step in the separation of complex mixtures of cellular components prior to downstream protein characterization using mass spectrometry (MS). Such tasks have become particularly challenging in the case of banana but also some other fruit tissues. To develop a representative protein map it is necessary to prepare biological extracts rich in protein species, which should reveal a wide range of molecular masses and isoforms over the 2D gel, and to remove a large amount of interfering substances, which in case of plant tissues represent polyphenols and polysaccharides.

Beside the difficulties in removal of polyphenol and polysaccharides, there is also a cumbersome issue with expression levels of protein in the plant material, which is influenced by various environmental stimuli (developmental stage, stress, etc). The problem with the low abundant proteins is that their presence in 2D map can often be beyond the limit of detection in standard proteomic, immunoblot, or even visualizing techniques. In contrast to that, the problem with high abundant proteins is that they can mask detection of less abundant

proteins. One possible method to solve the problem of dynamic concentration ranges is the usage of combinatorial peptide ligand libraries (CPLLs). CPLL was already described as the "Troyan Horse" in deep discovery proteomics and was shown to be a valuable method in analysis of low abundant proteins[3].

CPLLs represent mixtures of short peptides with different lengths (usually four to six amino acids) which are attached to solid beads. When proteins from the biological extract are exposed to this ligand library under large overloading conditions, each bead with affinity to an abundant protein will rapidly become saturated, and the remaining excess will stay unbound. In contrast to that, those low abundant proteins from the same extract will saturate the beads with corresponding peptides so they will be captured in increasing amounts. Because of the variations in allergen expression levels, detection and analysis of those proteins can be improved by using the CPLL approach in the development of representative 2D protein maps.

In this report, we are showing experimental solutions for the analysis of banana pulp proteins based on six amino acid CPLLs treatment for successive 2D PAGE, 2D PAGE/MS, and immunoblot analysis for the detection of novel banana fruit allergens.

Methods: Banana protein extract was prepared according to Nikolic et al (4). The protocol for obtaining concentrated banana proteins was optimized by combining salting out effect of ammonium sulphate (80 % saturation), followed by CPLL treatment. Removal of interfering substances (salts and pigments) was achieved by using TCA/acetone protein precipitation. Isoelectrofocusing of total banana proteins was performed under denaturating conditions using immobilized pH gradient (IPG stripes pH 3-10 NL, Serva, Germany). After separation in the first dimension (isoelectric point), the proteins were resolved according to molecular mass in the second dimension (4-12% Bis-Tris Gel, Novex, USA). 2D protein maps were visualized by stained with Coomasie Brilliant Blue (0.1%, CBB) dye for further MS analysis, or transferred onto nitrocellulose (NC) membrane for the immunoblot development. IgE reactivity of resolved banana proteins was detected by patients sera (n=12) in immunoblot.

Protein spots from 2D maps were treated with 10 mM ditiotreitol (DTT) and 55 mM iodoacetamide (IAA), and digested using sequencing grade porcine trypsin (Promega, Pierce), prior to MALDI TOF analysis.

Results: Banana proteins concentrated by ammonium sulphate were normalized by CPLL treatment. 2D PAGE protein map of the banana fruit extract revealed more than 200 clearly visible protein spots after CBB staining. Detected proteins showed isoelectric points in the range of pH 3-10 and molecular masses in the range of 14-116 kDa. In 2D immunoblot strong IgE reactivity to banana protein spots with molecular masses about 70 kDa (4.5-5.5 pI), about 90 kDa (6-7.5 pI), and about 55 kDa (6.5-8 pI) were detected, which do not correspond to previously registered banana allergens.

Using MALDI TOF analysis of IgE reactive spots and bioinformatic tools (Mascot), Mus a 2, Mus a 4, and Mus a 5 allergens have been identified.

Conclusions: We developed a reproducible protocol for the separation of banana proteins in a high resolution 2D map with equally distributed both high and low abundant banana fruit proteins. Maximizing the presence of different protein spots on 2D map and minimal streaking showed that this method overcomes problems of large amounts of starch, pectins, and polyphenolic compounds in the sample, and provides a convenient way to study different protein species that are present in banana fruit pulp.

This method provides sufficient amounts of proteins on 2D map for downstream analyses, which was confirmed by immunoblot and MS analysis in discovering potentially novel banana allergens.

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Fatty acids change the reactivity of the human serum albumin Cys34 thiol group

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Human serum albumin (HSA) is the most abundant plasma protein and with free Cys34 thiol represents a significant pool of the anti-oxidative thiol in human circulation. Crystallographic studies showed that accessibility of HSA Cys34 residue to oxidation was significantly changed when free fatty acids (FAs) were attached to HSA. Therefore, our aim was investigation of the impact of myristic acid(MYR, C14:0), palmitic acid(PLM, C16:0), stearic acid (STE, C18:0), oleic acid (OLA, C18:1), and fatty omega-3 polyunsaturated fatty acids from fish oil diet supplement EPA (C20:5) [(5Z,8Z,11Z,14Z,17Z)-5,8,11,14,17-icosapentaenoic acid] and DHA (C22:6) [(4Z,7Z,10Z,13Z,16Z,19Z)-docosa-4,7,10,13,16,19-hexaenoic acid] on reactivity of the Cys34 free thiol group.

Investigating of fatty acids content (bound to HSA) using GC analysis was time consuming process, and therefore we developed novel methodology utilizing quantitative TLC and flatbed scanner for densitometric quantification of FAs. Fast determination of the HSA saturation with FAs could be useful when estimation of the reactivity and redox capacity of the HSA-SH in circulation is performed, especially in some pathologies with elevated FAs (metabolic syndrome and diabetes).

Changes of thiol group reactivity before and after FAs binding was studied with DTNB (5,5'-dithiobis(2-nitrobenzoic acid)) reagent, at the concentration that represented pseudo-first order excess compared to HSA-SH. HSA changes were monitored using native PAG electrophoresis and fluorescence spectroscopy.

Time courses (0–10 min) of the reactions of FA-free HSA and FAs-bound HSA sulfhydryl group were monitored spectrophotometrically. Graphics obtained after linearization of kinetics data show that reactions followed pseudo-first order reaction kinetic. The values of rate constants ($k^{'}$) obtained for all FAs-bound HSA-SH (from 14.58×10^{-3} to 26.02×10^{-3} s^{-1}) were 2–3.5 times higher than for FA-free HSA-SH (7.52×10^{-3} s^{-1}). Among saturated long-chain FAs tested, the shortest FA, MYR, had the strongest effect. STE and OLA show similar effects on HSA-SH reactivity ($k^{'}$ values of 17.34×10^{-3} s^{-1} and 16.97×10^{-3} s^{-1}, resp.) although the effect of polyunsaturated

EPA was the highest ($26.02x10^{-3}$ s^{-1}). Between constants and k' values the Pearson product-moment correlation coefficient of 0.881 was found, suggesting that k' values were not random, but that some pattern existed. Except MYR, binding of all other FAs (especially unsaturated OLA, EPA and DHA) to HSA cause a decrease in the intensity of fluorescence at the peak wavelength. In addition, unsaturated FAs binding to HSA leads to the shift of the peak to the left. Rate constants for the HSA isolated from patients' serum were $12.0x10^{-3}$ to $17.5x10^{-3}$ s^{-1}, and FAs saturation ranging from 0.33 to 1.46 mol FAs per 1 mol of HSA.

Overall, FAs binding to HSA lead to the increase of Cys34 thiol group reactivity, and that reactivity of Cys34 thiol group depends on the type of FAs loaded to HSA, i.e. on the changes of the conformation of HSA molecules after FAs binding. This finding has an important implication for possible modulation of Cys34 thiol group reactivity (i.e. its scavenger capacity and antioxidant property) by FAs as a supplement.

Binding and Molecular Dynamics Studies of Phycocyanobilin with Human Serum Albumin

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Phycocyanobilin (PCB) is chromophore found in blue-green alga and cyanobacteria Spirulina. PCB has open-chain tetrapyrrole structure, similar to bilirubin, but with different conformation in aqueous solution and higher solubility, it can act as a potential ligand for Human serum albumin (HSA) protein.

HSA is the major soluble and the most abundant protein in blood plasma. Extraordinary acceptor capabilities for a wide variety of molecules make it an important tool in the development of novel therapeutic agents, and for determining pharmacokinetic behaviour of drugs. HSA is a monomeric, heart-like shape, helical protein consisting of 585 natural amino acids, and has a molecular mass of 66.5 kDa. With 67% α-helix and no β-sheet secondary structure elements, protein contains three homologous domains, each divided into two subdomains, A and B[1], and the overall structure is stabilized by 17 disulfide bridges.[2] Multiple ligand-binding sites have been reported for these subdomains. The two primary drug binding sites 1 and 2, first characterized by Sudlow et al.,[3] are located in subdomains IIA and IIIA. A third binding pocket within subdomain IB (site IB) has recently been identified as the primary binding site of a bilirubin photoisomer[4] and hemin[5].

In the current study, we used molecular docking calculations to find the best binding site and to analyse HSA-PCB as well as HSA-bilirubin interactions at molecular level. Additionally, we investigated conformational changes and influence of myristic acids (MYR) and other different ligands from PDB database on PCB binding to HSA. Further, for several HSA–PCB complex MD simulations were performed in aqueous solution in order to explore the stability and dynamic properties of the binding site(s).

Molecular modeling of the PCB/bilirubin–HSA complex was performed using the Protein Data Bank crystal structure of HSA (PDB ID: 1BM0). Additionally, the influence of conformational changes in protein as well as influence of bound fatty acids or other ligands on PCB binding to HSA was examined. To find the HSA binding sites with the best binding energy six different docking studies were conducted, with all possible protonation states of bilirubin and PCB ligand. Regardless of protonation state, two proposed HSA binding sites were found (IB and IIA). Both binding sites adopt L-shaped geometry, and phycocyanobilin ligand structure in M conformation.

Despite conformational changes of HSA, this two binding sites of phycocyanobilin are still the most energetically favorized binding sites although the estimated binding energies are somewhat influenced by protein conformation. Further docking studies showed that presence of myristic acid on all HSA binding sites except IB and IIA site does not influence phycocyanobilin binding to IB and IIA site. On the other hand, presence of different ligands prevent binding of PCB at IB binding site, but depending of molecule size it is possible to accommodate ligand and PCB at IIA binding site.

Initial structures of the proteins for molecular dynamics (MD) simulations are obtained from above mentioned docking study. All MD calculations were performed with NAMD 2.9 program[6] using the CHARMM27 all atom force field.[7] All starting structures were with ions added to counter the total charge of the protein. After minimization and equilibration phase, system was set to 0.3 μs production run in NPT ensemble using the Langevin piston pressure control at 310 K and 1.01325 bar with Particle Mesh Ewald (PME) method applied for a complete electrostatic calculation.

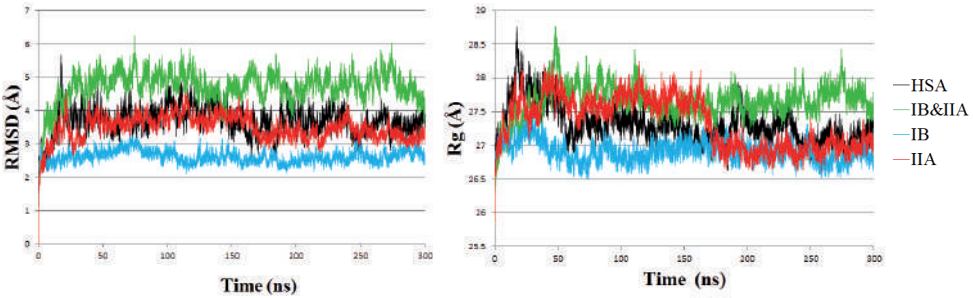
To evaluate the stability of the lowest docked energy protein complex, the properties were examined by means of root mean square deviation (RMSD), root mean square fluctuation (RMSF) and radius of gyration (Rg) of the protein. All data was calculated in program VMD[8] using in-house made tcl scripts.

The RMSD values of atoms in free HSA and HSA-PCB complexes with respect to initial structures were calculated along the whole trajectory and shown in Fig. 1. RMSD of all four systems reaches equilibrium and oscillate around an average value after 1500 ps simulation time. Complex with ligand bound to the IB site has the lowest RMSD value, whereas the biggest one arises from structure with PCB bound to both sites. RMSD value of HSA with ligand bound at IIA site shows little deviation in comparison to the whole protein, but with smaller value.

Local protein mobility was analysed by calculating the time-averaged RMSF values for all amino-acids of the free HSA protein and HSA-PCB complexes. The general profiles of atomic fluctuations were found to have lower values in binding sites, indicating that binding of PCB contributes to lower mobility of amino-acids in binding site.

In the present MD studies, we determined the radius of gyration (Rg) values of free HSA and all three HSA-PCB complexes as shown in Fig. 1. In all systems, Rg values were stabilized at lower value than initial except for two-ligand bound system. The radius of gyration of HSA and HSA with one PCB ligand bound is approximately similar to each other which clearly indicate that there are moderate conformational changes during the simulation, which is not case with two ligands bound to the HSA.

It is interestingly to note that by observing RMSD and Rg graphs there are two plots in system with PCB bound to IIA binding site. This could indicate possible allosteric effect of PCB binding to the HSA.



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Cold stability of proteases

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Proteolytic enzymes play fundamental roles in numerous physiological processes. Proteases are among enzymes with widest applications in food processing, pharmaceutical industry and detergent industry (they comprise around 60% of total enzyme production in the world) [1,2]. The main limiting factor for usage of proteolytic enzymes in industry is their thermal stability, both at low and elevated temperatures [3]. There is a common belief that proteases undergo inactivation due to autolysis during cold storage in solution, so major manufacturers recommend to store them in conditions far from optimal, with significant loss after each freeze-thaw cycle [4]. However, we could not find novel literature dealing with proteases' cold stability, especially published in recent decades, in which elucidation of the mechanism and understanding of the cold denaturation phenomenon are in the focus. An interesting question arises out of this problem. Could the cold storage of a protease in conditions far from its optimal conditions cause an increase of inactivation due to cold denaturation to a greater extent than the loss of activity due to autolysis?

Here we reported a comparison of primary structure instability (caused by autolysis) and tertiary structure instability (caused by cold denaturation) for four model proteases (ficin, papain, trypsin and fig collagenase). Due to the limitations of spectroscopic methods for investigating protein samples in frozen state, we employed approach of repeated freeze-thaw cycles to study cold stability of proteases. Activity of model proteases was monitored with specific assays after each freeze-thaw cycle. Autolysis was quantified in colour-binging assay and monitored by reverse-phase HPLC. Secondary structure changes after repeated freeze/thaw cycles of model proteases were determined by Fourier transform infrared spectroscopy (FT-IR). Aggregation of misfolded proteins was monitored by analytical SEC-HPLC and electrophoresis.

Among 4 tested proteases, fig collagenase showed exceptional pH and thermal stability [5]. During cold storage, no activity loss, autolysis and changes of overall structure were detected.

In the case of trypsin, papain and ficin we detected dramatic activity loss after 6 freeze-thaw cycles (75% for papain, above 40% for ficin and around 60% for trypsin stored in acidic conditions) with very modest autolysis (less than 10% of proteins was lost due to autoproteolysis), suggesting that dramatic inactivation of proteases can not be attributed to autolysis.

On the other hand, after 6 freeze/thaw cycles these proteases lost parts of their native secondary structure elements (mostly α-helix and unordered structures) in favor of β-sheet structure, especially intermolecular β-sheet content increased indicating the presence of secondary structures necessary for aggregation. Several authors have reported an increase of the β-sheet content in cold or acid denatured proteins compared to native ones, sometimes followed by formation of aggregates [6,7], leading to conclusion that detected changes in secondary structures of ficin, papain and trypsin were the consequence of cold denaturation.

Furthermore, in the case of trypsin, the protease of the greatest commercial importance used in this study, we proposed altered cold storage conditions in order to avoid cold denaturation and to limit autoproteolysis. Slightly alkaline pH value (optimal pH for the activity) and the usage of cryoprotective additives that are known to be preferentially excluded from protein surface [8], thus favoring its folded state (glycerol and lysine) led to preservation of trypsin tertiary structure, while the presence of lysine limited autolysis probably by occupying enzyme's substrate-binding pocket. Nevertheless, trypsin stored at pH 8.2 with the addition of glycerol or lysine was as efficient as untreated trypsin in the trypsin mass fingerprinting analysis of BSA, suggesting that the cold storage of trypsin in slightly alkaline conditions with the addition of cryoprotectants could prolong its shelf life.

Despite of general belief that the activity loss detected upon the cold storage of proteases happens as a consequence of autoproteolysis, in this work it is undoubtedly shown that upon the cold storage proteases underwent structural rearrangements and aggregation that correspond to other cold denatured proteins, rather than autoproteolysis. Studying cold stability of industrially important proteases could lead to development of better preservation methods (the usage of cryoprotectants) and prolonged shelf life as confirmed in the case of trypsin for sequencing.

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N-glycome of membrane proteins and receptors for insulin and insulin-like growth factors, isolated from human placenta from different (patho)physiological conditions

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Introduction

Membrane proteins have numerous functions such as cell-cell communication, adhesion, signal transduction etc. Most of them are glycosylated and N-glycosylation plays important role in the formation of three-dimensional structure and function of membrane proteins. It was shown (for EGFR, NGFR, IR) that complete blocking of the N-glycan synthesis leads to formation of a receptor that is unable to reach membrane surface and, therefore, remains in the cytoplasm. Systemic profiling of human serum N-glycome in health and disease is an ongoing process, with few thousands of samples analysed so far. It was shown that N-glycosylation of serum proteins alters during aging and alteration in the N-glycan content of some of them has already been related to different diseases. Investigation on N-glycome of membrane proteins started recently and the data are still scarce. Detergent (necessary for protein solubilisation) incompatibility with numerous analytical methods, especially those used for structural analysis of glycans, is probably the main reason for rare literature data regarding glycosylation of membrane proteins.

Receptors for insulin (IR) and the type 1 insulin-like growth factors receptor (IGF1R) are transmembrane tyrosine kinases with high degree of homology (in some domains even 80%). Ligand binding (insulin, IGF-I or IGF-II) triggers kinase activity, leading to autophosphorylation of a receptor and activation of receptor substrates. Signalling cascade that follows ligand binding to IGF1R leads to different mitogenic processes, whereas IR activation induces metabolic response. Together with IR and IGF1R, the family of IGF receptors includes the type 2 insulin-like growth factor receptor (IGF2R). IGF2R, also known as cation-independent mannose-6-phosphate receptor (since it binds mannose-6-phosphate), is a transmembrane glycoprotein which is included in intracellular trafficking of lysosomal enzymes. Binding of IGF-II to IGF2R leads to degradation of this peptide. There are new findings indicating IGF2R involvement in signal transduction via IGF-II. IR, IGF1R and IGF2R are abundantly present in placenta, where they play important roles in placental development and functions.

Human placenta was used as a model tissue in this PhD thesis. Placenta grows and differentiates to fulfil different needs of a foetus, so its structure and function changes during gestation. Knowing that proteins are responsible for placental biological functions, it was assumed that during gestation, changes could occur in the expression and/or function of membrane proteins. In the experimental part, the type of N-glycans commonly found attached to membrane glycoproteins was examined, which resulted in an overview of the membrane N-glycome of human placental proteins. The susceptibility of membrane N-glycome to individual variations and to the effect of maternal/women age was investigated as well. In a separate set of experiments, the influence of the gestational stage on membrane N-glycome was examined. And finally, knowing that altered glycosylation is often related to altered function, and that altered function of one or more proteins can cause a disease, potential changes in membrane N-glycome were analysed in pathological pregnancies (preeclampsia) and in pregnancies complicated by a pathology of a mother (diabetes) (*Robajac et al., Journal of Medical Biochemistry 2011, 31:205-210*). The impact of aging, gestation and pathology on the type and abundance of different N-glycans on receptors belonging to the IGF system was investigated in parallel. The aim was to resolve whether changes on the entire membrane N-glycome coincide with changes on specific membrane glycoproteins, such as IR, IGF1R and IGF2R.

Material and methods

Membrane proteins were solubilised from the first (n=160) and third trimester placentas (n=30) obtained from women of different age, as well as from placentas obtained from pathological pregnancies (preeclampsia n=14; diabetes mellitus type 2 n=6). Alterations in membrane N-glycome were analysed using DNA sequencer-aided fluorophore-assisted carbohydrate electrophoresis (DSA-FACE). Prior to DSA-FACE analysis, N-glycans were enzymatically cleaved and labelled using 8-aminopyrene-1,3,6-trisolfonic acid. Solubilised membrane proteins were also analysed using lectin blot. The type and the structure of N-glycans present on the receptors of the IGF system were analysed using lectin affinity chromatography with radioactively labelled ligands.

Results

Membrane N-glycome of human placental glycoproteins was reported for the first time in this PhD thesis, identifying eleven N-glycans as the most abundant in all samples. Results have shown that maternal age had no impact on membrane N-glycome. On the other hand, there was an increase in the content of paucimannosidic N-glycan structures, NA2F and NA3F complex N-glycans, and a decrease in the content of NGA2, max4, NA2, max6 and NA2FB along the gestational period. A gestational increase in the amount of core fucosylated and multiantennary N-glycans was found, followed by a decrease in bisecting GlcNAc moieties. Pathological conditions, like preeclampsia, lead to an increase in the amount of smaller

and incomplete N-glycans (such as paucimannosidic structures, NGA2 and max4), and a decrease in the content of more complex N-glycans (such as NA2, max6 and NA3F).

Investigation on receptors demonstrated that maternal age had no effect on the content of N-glycans present on IR and IGF2R. On the other hand, IGF1R in the first trimester of pregnancy, obtained from placenta of younger women, had higher content of biantennary N-glycans and lower content of tri- and tetraantennary N-glycans compared to older women. The amount of total fucosylated, core Fuc and α2,6-sialo-N-glycans on IR decreased during gestation. Gestational decrease of total fucosylated and α2,6-sialo-N-glycans and an increase of core Fuc was found on IGF1R. Opposite to IR and IGF1R, gestational increase of total and core fucosylated N-glycans was found on IGF2R. Pathological conditions did not significantly change N-glycosylation pattern of IR, IGF1R and IGF2R. Ligand- and lectin-binding assays demonstrated that the presence of a specific carbohydrate on IR (Sia), IGF1R (Fuc) and IGF2R (Fuc) was crucial for their interactions.

Conclusion

Data obtained in this PhD thesis indicated significant gestational differences in nine out of 11 most abundant N-glycans present in human placental membrane, suggesting strong gestational impact on placental N-glycome. It was also found that changes in the entire membrane N-glycome were not necessarily related to changes in the N-glycan content of a specific membrane glycoprotein, such as receptors of the IGF system. This PhD thesis is the first report on human placental membrane N-glycome. The final conclusion, summing up all data obtained, is that N-glycosylation of proteins is developmentally regulated (*Robajac et al., Mechanisms of ageing and development 2014, 138:1-9*).

Characterization of fullerenol/doxorubicin nano composite, its cytotoxicity in malignant cell lines along with cardio- and hepato- protection in rats

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Although it has been sixty years since the discovery of doxorubicin (DOX), its presence in chemotherapy of large numbers of malignant diseases is still inevitable. Its use in clinical practice is limited by systemic toxicity with special emphasis on the damage to the heart and liver as well as the phenomenon of resistance to antibiotics alone. In order to reduce its toxic effects on vital organs, dose reduction through a new formulation of the drug is offered as a reasonable solution to the problem. The mechanism of doxorubicin toxicity is primarily based on oxidative stress. One way to reduce the damage caused by oxidative stress is to use antioxidant agent. Fullerenol is one such molecule that is proved in many previous studies to be a good scavenger of free radicals. As a molecule, fullerenol has the size 1nm, while in aqueous solutions is present in the form of polianionic particles (FNP) with an average size of 10nm - 60nm. Its negative charge and large surface represents the ideal nano platform that has the ability to ''stick'' to themselves planar and positively charged doxorubicin.

In our research group has been designed a new formulation of the drug in the form of nano FNP / DOX nanocomposite. Nanocomposite was characterized by different physico-chemical and nano methods. DLS was used to determine the size distribution of particles in aqueous solution. It was observed that the particle size was not changed compared to the pure aqueous solution of fullerenol and that is in the range of 10nm - 60nm. The distribution was measured in cell media and at different temperatures as well as at different pH values, and in different time intervals, and no significant changes were observed in the distribution of nanocomposite, which indicated its stability. Measurements made on the TEM as well as AFM were in agreement with measurements on the DLS. Zetta-Sizer demonstrated that the charge of the particles changed from -40mV, when in the solution was present only to fullerenol to + 40mV after forming FNP / DOX nano composite.

After the characterization of FNP/DOX nano composite, it has been continued with testing its biological effects and was found out to have a significantly better cytotoxicity at the same dose as well as in smaller doses in comparison to a commercial drug doxorubicin.

The cytotoxicity was indicated by MTT test on the two human malignant cell lines (MCF-7 and MDA-MB-231). Flow cytometry showed that uptake of doxorubicin was significantly increased in the malignant cells when it was in the form of FNP/DOX nano composites than when it is applied alone. Increased cytotoxicity of FNP/DOX nanocomposite compared to doxorubicin alone is explained by a greater amount of doxorubicin which enters the cells in the form of a composite as well as the high sensitivity of malignant cells compared to healthy ones.

The cytotoxicity of the FNP/DOX nanocomposite has also been tested on HL-1 cardiomyocytes which is continuous line of mice. It was obtained a significantly better effect of the cell survival in comparison with doxorubicin applied alone, suggesting a protective effect of fullerenol within FNP / DOX nanocomposite.

After *in vitro* tests, the effects of FNP/DOX nanocomposite were tested *in vivo*. Healthy adult male Wistar rats were divided into six groups and treated i.p.: The control which received saline, 0.125mg / kg FNP group, a two doxorubicin groups, 2 mg / kg DOX, and 4 mg / kg DOX, and two nanocomposite groups, 2 mg / kg of FNP / DOX and 4mg / kg of FNP / DOX. After 24 hour treatment, the rats were sacrificed by urethane anesthesia and organs were collected for further analysis, TEM and qRT-PCR. By qRT-PCR were monitored gene expression levels of the antioxidant enzymes: catalaze and MnSOD, as well as genes involved in apoptosis: BCl-2 and Bax, bearing in mind that in healthy tissues doxorubicin induced oxidative stress and the cells die through programmed cell death. Ultrastructural analysis was used to monitor changes within the tissue as well as subcellular changes of cardiomyocytes and hepatocytes. The results of both types of analysis are in agreement with each other indicating that FNP / DOX nanocomposite is less toxic to the tissues in comparison to doxorubicin applied alone. Namely, in groups where doxorubicin was applied alone at ultrastructural level was observed lipid peroxidation in the form of myelin figures, damaged mitochondrial cristae, pyknotic nuclei and edematous cells as signs of apoptosis, damaged discus intercalatus. These changes were either absent or were in a much lower extent present in the groups treated with FNP/DOX nanocomposite. As for the level of mRNA for catalase and MnSOD, in groups treated with FNP/DOX nanocomposite were decreased with respect to the group treated only with doxorubicin, suggesting a reduction of oxidative stress. Further, Bax and BCl-2 resulted in increased levels of mRNA expression in all treated groups compared to the control. The role of BCl-2 in the cell is to prevent apoptosis by inhibiting Bax in a way that Bax and BCl-2 make dimers. Bearing in mind that the results showed overexpression of BCl-2 it could be concluded that the cells resist apoptosis.

Overall results imply that the novel nano formulation of doxorubicin with fullerenol has the great potential for use in clinical trials.

Mixed-mode resins: taking shortcut in downstream processing of raw-starch digesting α-amylases

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Bacillus licheniformis 9945a α-amylase (*Bli*Amy) has been described as potent enzyme for raw starch hydrolysis. Starch represents an inexpensive source for production of glucose, maltose syrups and fructose which are widely used in food industries. Regarding energy costs, effective utilization of natural resources and viscosity problems, direct hydrolysis of raw starch below the gelatinization temperature by using raw-starch-digesting enzymes, such as α-amylase is desirable. In spite of the extensive studies concerning the structure and thermal properties of *B. licheniformis* amylase and the numerous reports in the literature referring to the molecular mechanism of irreversible thermoinactivation, little attention has been paid to its enzymological characterisation. Detailed knowledge about subsite architecture of *B. licheniformis* amylase is scarce. No report on kinetics and mode of action of this industrially important enzyme can be found in the literature especially when raw starch is used as a substrate. For mechanistic studies enzyme preparations of high purity are required and improving downstream processing is very beneficial. *Bli*Amy was produced using optimized fed-batch approach in defined media and significant overexpression of 1.2 g L^{-1} was achieved. These amylases have exposed tyrosine and tryptophan residues as part of their surface binding sites. Mixed mode Nuvia cPrime™ resin is tested as improvement of the downstream processing of raw starch digesting amylases aiming at exploiting hydrophobic patches at their surface. This resin combines hydrophobic interactions with cation exchange groups. Presence of salt facilitates hydrophobic interactions while ion-exchange groups enable proper selectivity. Surface response methodology was used to optimize binding and eluting conditions of *Bli*Amy. This single step procedure enables simultaneous concentration, pigments removal and purification of amylase with a yield of 96% directly from fermentation broth.

Molecular forms of IGF binding protein 2 and their presence in various pathophysiological states

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Insulin like growth factor (IGF) system comprises of two peptides (IGF-I and IGF-II), a family of six IGF binding proteins (IGFBP-1 to IGFBP-6) and their proteases, two types of IGF receptors (IGF receptors type 1 and 2 - IGF-1R and IGF-2R), as well as receptors which are not strictly part of this system, but share functional similarity with IGF-1R (insulin receptor - IR and the hybrid receptor - IR/IGF-1R). IGFs function as mitogens which stimulate growth, division and differentiation of cells, and possess anti-apoptotic activity. They exert their biological actions by binding to IGF-1R, IR and the hybrid receptor. The concentration of free peptides in the circulation and in tissues is tightly controlled by binding to IGFBPs, whose affinities for IGFs are higher than those of receptors. In order to liberate free IGF peptides, IGF/IGFBP complexes must be cleaved by proteases which partially degrade IGFBPs, reducing their affinity for IGFs. Besides IGF binding, IGFBPs have the potential to act independently, exerting pro- or anti-apoptotic activity on cells, depending on the type of IGFBP and the type of the tissue.

IGFBP-2 (36 kDa) is the second most abundant binding protein in the peripheral circulation, after IGFBP-3, and its average concentration is 370-550 ng/ml. Its concentration does not show significant daily fluctuations, accept long-term rise as a consequence of caloric and/or protein restriction. Besides binding IGF peptides, it can form complexes with another protein in the circulation, alpha-2-macroglobulin (α2M), which is the original result of the PhD thesis (*Šunderić et al., Protein J (2013) 32:138-142*) . The physiological role of this association is not elucidated yet.

IGFBP-2 can be found in three main forms in the circulation: as a native molecule - monomer, in the form of frgaments of different masses (4-21 kDa), and as a complex with α2M. When the concentration of IGFBP-2 in the circulation is determined, the total IGFBP-2 is usually considered, without distinction between its forms. However, since different forms may exert different roles and their performances may depend on the size of the molecule, it is important to investigate and know their distribution. Particular IGFBP-2 forms change in certain pathophysiological states and investigation of the factors which alter the equilibrium is one of the topics of this thesis.

During fetal development, IGFBP-2, besides IGFBP-1, is the most abundant IGF binding protein in the circulation. In neonatal period its concentration falls and IGFBP-3 becomes the major IGFBP, followed by IGFBP-2. During puberty, the concentration of IGFBP-2 continues to fall, but with aging (after 30 years) it begins slowly to rise, reaching maximal concentration after the age of sixty. There are a lot of controversial results on the impact of IGFBP-2 on the aging process. Higher levels of IGFBP-2 in the circulation were shown to protect against obesity and diabetes type 2 whereas, on the other hand, they were recognized to induce weakness of the musculoskeletal system and the propagation of cancer. These contradictory data imply that IGFBP-2 possesses tissue-specific actions, affecting differently a number of tissues. Experimental results of this PhD thesis demonstrated that the quantity of IGFBP-2 changes with ageing, but not in the same way for all molecular forms. The amount of IGFBP-2/α2M complexes falls, whereas the amount of IGFBP-2 monomer and fragments rises (*Šunderić et al., Exp Gerontol (2014) 58:154-158*). Thus, the concentration of "free" IGFBP-2 increases with age and IGFBP-2 is more readily proteolysed, releasing IGFs to act in an anabolic manner. IGFBP-2 can also perform its IGF-independent activity more intensively in elderly people.

Although mechanisms of the IGFBP-2 action are not fully understood, it is known that IGFBP-2 plays important role in metabolic processes, especially in those connected with age-related diseases. It is known that greater serum concentration of IGFBP-2 protects against obesity and diabetes type 2. On the contrary, decreased IGFBP-2 concentration is followed by impaired lipid profile in the circulation. Thus, the next task in the PhD thesis was to monitor the influence of various physiological factors on the distribution and quantity of specific IGFBP-2 forms. The effect of the following parameters was examined: sex, intensive physical activity (*Nedić et al., J Sport Health Sci (2015), accepted article*), moderately increased concentration of glucose, triacylglycerols and cholesterol. The results obtained showed no difference in distribution and quantity of the particular IGFBP-2 molecular forms between healthy persons and those with increased concentration of glucose or lipids.

IGFBP-2 is being under investigation as a tumor marker. It was shown that serum concentration of IGFBP-2 is increased in almost every type of cancer and it advances with cancer progression. Although IGFBP-2 does not seem to play a role in early stages of tumorigenesis, it stimulates cell motility (in IGF-independent mode), helping in dissemination of tumor and metastasis. The expression of specific IGFBP-2 molecular forms in eight most common cancer types (liver, pancreas, colon, lung, prostate, breast, cervix and ovary) was examined in this PhD thesis. The concentration of monomer IGFBP-2 was found elevated in all types of cancer, while the concentration of IGFBP-2/α2M complexes was elevated in some types of tumors (pancreas, colon, breast and ovaries) and reduced in others. The most important conclusion drawn from these experiments was that in some tumors IGFBP-2/α2M complexes limit IGFBP-2 availability (and activity), whereas in others they fail to do so. This mechanism may participate in the regulation of tumor cell motility and metastasis, although at the moment there is no experimental proof (*Šunedrić et al., Exp Mol Pathol (2015) 98:173-177*).

Since α2M is heavily glycosylated molecule and the glycosylation pattern may change due to tumorigenesis, the last part of the PhD thesis was dedicated to investigation of α2M glycosylation in patients with colorectal carcinoma. Lectin microarray was used and it was found that an overall degree of glycosylation of α2M was increased in patients and that patients' α2M had higher amount of multi-antennary complex type N-glycans (*Šunderić et al., DOI:10.1002/bab.1407)*. Structural changes in α2M carbohydrate moiety can reflect in altered interaction of α2M with IGFBP-2 (decreased affinity?), affecting the entire equilibrium of ligand binding and cellular response. Glycans in α2M are currently being under investigation as potential glyco-markers for colon cancer.

Old herbs for the new therapy of type 1 diabetes

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Introduction

Type 1 diabetes (T1D) is an autoimmune inflammatory disease, clinically manifested by hyperglycemia that develops as a consequence of destruction of insulin-producing pancreatic beta cells. Since there is no cure for T1D, the focus of novel experimental therapeutic approaches is finding an immunosuppressive drug that will selectively target autoimmune response and preserve innate immunity toward external pathogens. Vast number of studies has linked the consumption of food and natural products of plant origin rich in biophenols with protection against chronic inflammatory diseases. *Origanum vulgare*, besides being a spice, is also a source of natural antioxidants and exerts anti-fungal, anti-bacterial and anti-inflammatory effects. Goal of this study was to determine effects of three different oregano extracts: aqueous (AOE), methanol (MOE), and ethyl acetate extract (EAO) on T1D development.

Materials and methods

The extracts were provided from colleagues at Department of Chemistry, University of Ioannina, Greece. The extracts were administered to C57BL/6 mice in dose of 5 mg/kg per day in prophylactic regimen for 10 consecutive days. Diabetes was induced by administration of multiple low doses of streptozotocin (i.e. 40 mg/kg bw for five consecutive days). Evaluation of effect of extracts on T1D was performed by monitoring glycemia and insulin (ELISA) and *ex vivo* assessment of insulitis (histochemistry) and immune response (flow cytometry, ELISA, real-time PCR) within lymph node cells and mononuclear cells that infiltrate pancreatic islets. For assessment of antioxidant status of pancreas, activity of antioxidant enzymes (superoxide dismutase, catalase, glutathione peroxidase) was measured. Effect of extracts on apoptosis of beta cells was measured using caspase 3 assay.

Results

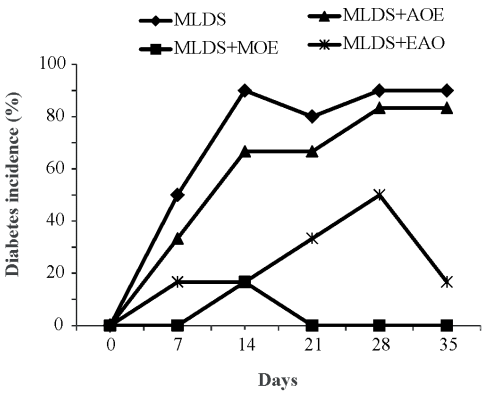

Fig. 1. Diabetes incidence in C57BL/6 mice treated with oregano extracts.

While prophylactic AOE therapy had no impact on diabetes prevention, MOE and EAO significantly reduced diabetes incidence (Fig. 1) and preserved normal insulin secretion. In addition, MOE scavenged reactive oxygen and nitrogen species and therefore alleviated the need for stimulation of antioxidant enzymes within the pancreas. As for the effect on immune system, MOE treatment specifically attenuated the pro-inflammatory response mediated by T helper 17 cells and enhanced anti-inflammatory T helper 2 (Fig. 2) and T regulatory cells through the impact on specific signaling pathways and transcription factors. Importantly, MOE preserved beta cells from *in vitro* induced apoptosis *via* blockade of caspase 3.

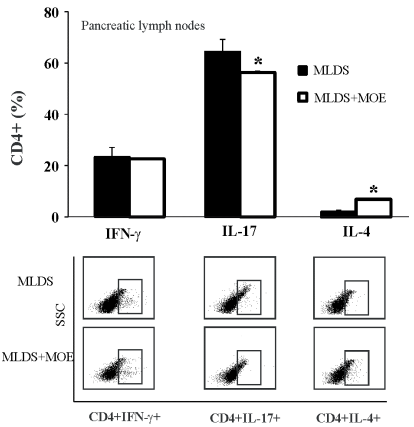

Fig. 2. The effect of MOE on T helper cell distribution in pancreatic lymph nodes.

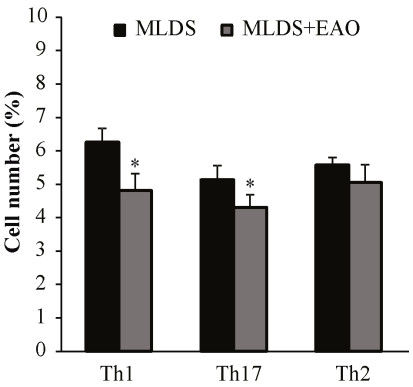

Fig. 3. The effect of EAO on T helper cell distribution in spleen.

In contrast to MOE which exerted its effect only on T lymphocytes, treatment with EAO suppressed macrophage and T lymphocyte-mediate immune response (Fig. 3).

Conclusions

In conclusion, MOE and EAO exerted immunomodulatory and anti-diabetogenic effects, but the effects of MOE were more pronounced. What is more, its specific effect on adaptive immune response makes MOE a good candidate for selective immunosupression.

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