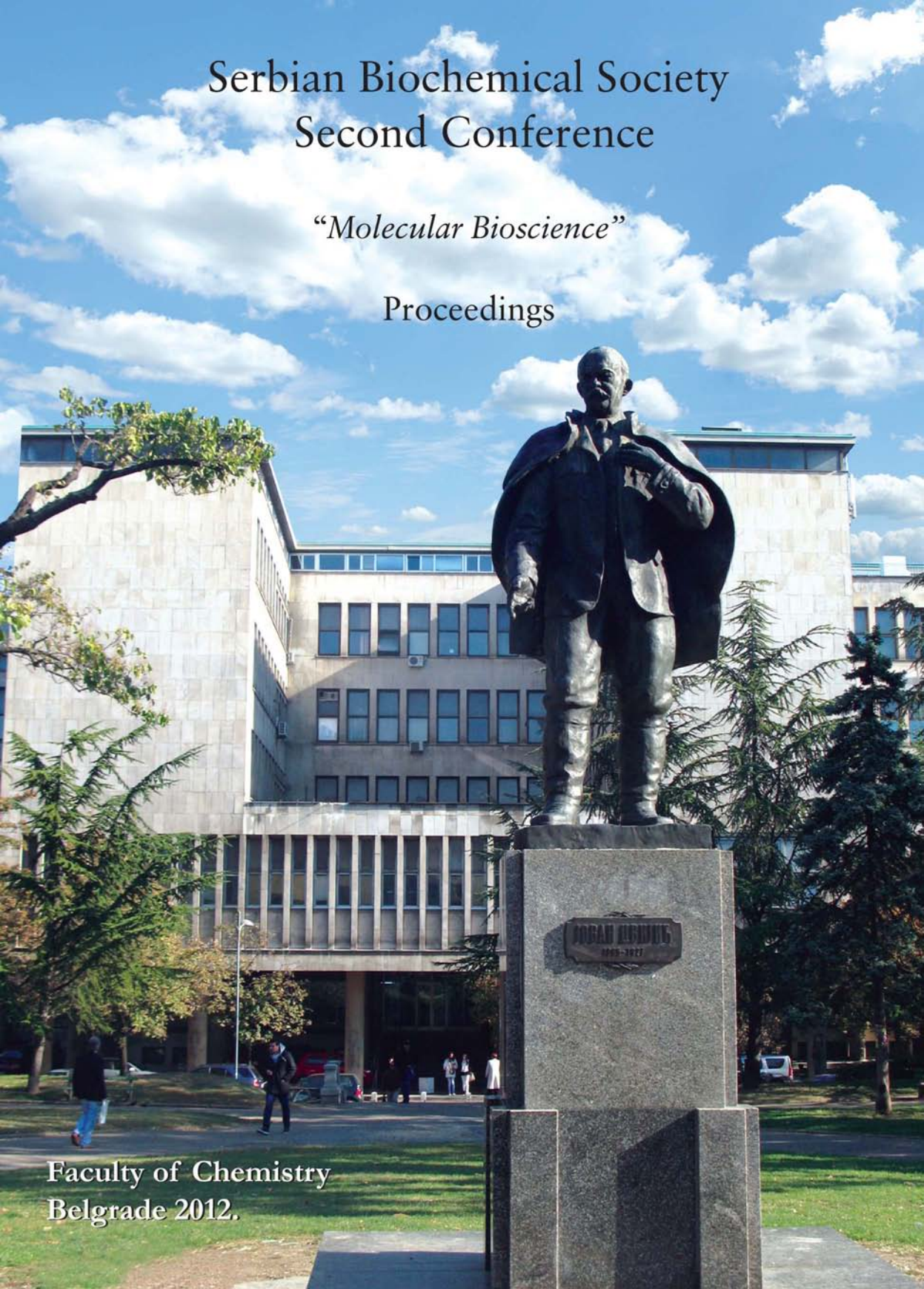
Serbian Biochemical Society
Second Conference

"Molecular Bioscience"

Proceedings



Faculty of Chemistry
Belgrade 2012.

Serbian Biochemical Society

Proceedings

Edited by: Mihajlo B. Spasić
Technical secretary: Jelena Nestorov
Cover and logo design: Aleksandra Nikolić - Kokić
Publisher: Faculty of Chemistry, Serbian Biochemical Society
Printed by: Colorgrafx, Belgrade

Serbian Biochemical Society Second Conference

University of Belgrade, Faculty of Chemistry.
9.11.2012. Belgrade, Serbia.

"*Molecular Bioscience*"

PROGRAM

10.00-10.10 Welcome messages from:

Prof. M. B. Spasi}
(President of the Serbian Biochemical Society)

Prof. B Jovan~i}evi}
(Dean of the Faculty of Chemistry)

10.10-10.55 *FEBS lecture*

Prof. Israel Pecht (FEBS Secretary-General)

Department of Immunology, The Weizmann Institute of Science, Rehovot, Israel

The Type 1 Fce Receptor
A Double -faced Immunoreceptor

10.55-11.10 Discussion

11.10-11.30 Short break

11.30-11.50 Tanja] irkovi} Veli~kovi}, PhD

Department of Biochemistry, Faculty of Chemistry, University of Belgrade, Belgrade, Serbia.

Protein digestion, immunopathologies and health

11.50-12.10 Ivanka Karad`i}, PhD

Department of Chemistry, School of Medicine, University of Belgrade, Belgrade, Serbia.

Topology of proteasomal core particle of *Haloferax volcanii* by chemical cross-linking, mass spectrometry and bioinformatics

12.10-12.30 Edvard T Petri, PhD

Department of Biology and Ecology, University of Novi Sad, Novi Sad, Serbia.

Application of structural biochemistry to the study of mechanisms of ion channel activation

12.30-12.40 Discussion

12.40-13.00 Short break

13.00-13.20 *SCS lecture*

Nata{a Bo`i}, PhD

Centre for Chemistry, Institute of Chemistry, Technology and Metallurgy, University of Belgrade, Belgrade, Serbia.

Cold enzyme hydrolysis of starch

13.20-13.40 Niko S. Radulovi}, PhD

Department of Chemistry, Faculty of Science and Mathematics, University of Ni{, Serbia.

Harnessing the biological activity of natural products: Isolation, synthesis and screening of biological/pharmacological activity

13.40-14.00 Marina Mitrovi}, PhD

Department of Biochemistry, School of Medicine, University of Kragujevac, Kragujevac, Serbia.

Regulation of apoptosis in various experimental models of diseases

14.00-14.30 Break with refreshments provided.

14.30-14.50 Nevena Grdovi}, PhD

Institute for Biological Research “Sini{a Stankovi}” University of Belgrade, Belgrade, Serbia.

CXCL12 and PARP-1 are potential key molecules in the promotion of β-cell survival and diabetes attenuation

14.50-15.10 Aleksandra Stankovi}, PhD

Institute of Nuclear Sciences “Vin~a”, University of Belgrade, Belgrade, Serbia.

Genetic basis of inflammation in human diseases

15.10- Discussion and concluding remarks

Foreword

Dear Colleagues,

It is my great pleasure to wish you warm welcome to the Second Conference entitled “Molecular Bioscience” organized by the Serbian Biochemical Society.

Second Conference of the Serbian Biochemical Society indicate that wish from the foreword of the First Conference “that it is beginning of continual work for many years to come!” have start to be truth. We have invited Secretary General of FEBS to be lecturer and eight from Serbia to present their state of art in the field they work as invitation for further co-operation. Their presentations are published in Proceedings. I express my gratitude to the members of governing board of Serbian Biochemical Society who suggested lecturers and to all of them who accepted invitation.

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

The Type 1 Fce Receptor A Double-faced Immunoreceptor

H. Péterfy[1], G. Tóth[3], A. Erdei[1,2] and I. Pecht[4*]

[1]Department of Immunology and [2]Immunology Research Group of the Hungarian Academy of Sciences at Eötvös Loránd University, Budapest, Hungary
[3]Department of Medical Chemistry, University of Szeged, Szeged, Hungary,
*[*4]Department of Immunology, The Weizmann Institute of Science, Rehovot, Israel*

Abstract

Peptides with the natural or modified sequences of the complement component C3a, (named C3a7 and C3a9) were shown to inhibit the type 1 receptor for IgE (FcεRI) induced degranulation of both mucosal and serosal type mast cells. We have demonstrated that phosphorylation of the β-chain of the FcεRI-complex and of Lyn, as well as the Ca^{2+}-response of RBL-2H3 cells were all decreased in the presence of these peptides. Investigation of the interaction of C3a9, (the more effective complement-derived peptide) with FcεRI now further clarifies its impact on the FcεRI coupling events. We found that the peptide binds to FcεRI on the surface of unperturbed RBL-2H3 rat cell-line and remains bound even after its aggregation by antigen as measured by confocal microscopy using Cy3-IgE and Bodipy 650/665-C3a9. As internalization of FcεRI is one of the events which are known to down-regulate mast cell response, we investigated the effect of C3a9 on this process. We found that during the time-interval where intracellular signalling steps are initiated (~5 minutes after receptor-clustering), the peptide significantly enhances the antigen-induced endocytosis of FceRI. Moreover, we demonstrate that peptide binding to the FceRI b-chain interrupts the FcεRI-β-chain association with both src kinases Lyn and Fyn. C3a9 was also found to inhibit the phosphorylation of ERK and p38. Although ERK is usually activated via the ras-raf-MEK pathway, our results show that C3a9 has no effect on the c-raf phosphorylation. Therefore we suggest that the complement-derived peptide inhibits ERK activation via an alternative route. Investigating the peptide's effect on the late phase response of bone marrow-derived mast cells we found that C3a9 decreases secretion of the inflammatory cytokines IL-6 and TNF-α. Altogether our results suggest that the C3a-derived peptides, inhibit both the immediate and the late phase response of mast cells as a consequence of its interference with the earliest steps of FcεRI-stimulus-response coupling.

Proceedings

Protein digestion, immunopathologies and health

Tanja Ćirković Veličković*

* *Department of Biochemistry, Faculty of Chemistry, University of Belgrade, Belgrade, Serbia*
e-mail: tcirkov@chem.bg.ac.rs

Diet-related diseases such as hypertension, atherosclerosis, diabetis, heart disease, osteoporosis, affect millions world-wide. Food digestion may positively affect human health via the release of bioactive components in the gut during digestion, but also exerts an influence on immunopathologies, such as food allergy and intestinal bowel disease. Bioactive molecules, such as peptides, micronutrients, phytochemicals, released by digestion, may have an action (positive or negative) on human health. The structure and composition of food influence the nature of bioactives released in the gut.
The structure of food depends on and can be modified by the food processing conditions. The evolution of food during digestion can be followed using *in vitro* digestion models and/or *in vivo* using different animal models or human volunteers. Detection of bioactives in the gut and peripheral blood gives further evidence of their resistance to digestion and reinforces their potential role on human health.
Controlling the release of bioactive compounds, macro- and micronutrients release, or appetite regulating compounds by digestion is of great importance for development of new innovative functional foods.

Health effects related to protein digestion

Bioactive peptides

Hydrolysis of dietary proteins releases free amino acids and peptides that may exert various potential bioactivities such as antihypertensive, antimicrobial, immunomodulatory and appetite regulation properties[1-3]. Bioactive peptides derived from milk proteins may play an important role in the prevention and treatment of metabolic syndrome and its complications via several mechanisms, such as the satiety response, the regulation of insulinemia levels and blood pressure, the uptake of free radicals, and alteration of the lipid profile[4, 5].

Food allergies

Resistance of protein sequences to digestion is considered to be a major factor in the sensitization phase of food allergy[6]. Hindrance of gastric digestion by elevation of the gastric pH, the therapeutic goal of anti-ulcer medication, was recently shown to trigger food allergy

via oral sensitization[7]. These studies indicate for the first time the important gate-keeping function of gastric digestion, both in the sensitization and the effector phases of food allergy. Thus, it is a general hypothesis that food allergens must exhibit sufficient gastro-intestinal stability to reach the intestinal mucosa where absorption and sensitization can occur. Based on this, investigation of the stability of proteins within the gastrointestinal tract provided prospective testing for the allergenic potential and one of the parameters used to distinguish food allergens from nonallergens. Various *in vitro* digestion models were developed to assess the stability of food allergens during digestion[8], but systematic evaluation of the stability of food allergens that are active *via* the gastrointestinal tract (GIT) is currently tested in traditional pepsin digestibility models[9].
Special attention is focused on genetically modified (GM) crops that should be carefully tested before entering the market, including a detailed analysis of allergenic risks of novel proteins[10]. The stability of a protein to digestion, as predicted by an *in vitro* simulated gastric fluid assay, is currently used as one element in the risk assessment process[9]. In this context, *in vitro* digestibility tests, cell-based assays, IgE-binding tests and analytical methods and profiling technology may provide useful information for the allergenicity assessment of GMOs.

Gut microbiota

Bacteria within the colon, the 'gut microbiota', play important functions such as energetic metabolism, proliferattion and survival of epithelial cells, and protection against pathogens. Chronic alteration of intestinal microbiota homeostasis could promote many diseases, including cancer [11]. The mechanisms by which bacteria may induce carcinogenesis include chronic inflammation, immune evasion, and immune suppression. The microbiota of the colon derive their energy from dietary compounds which escape digestion in the stomach and small intestine, and endogenous substrates e.g. mucins, secreted by the host. Protein reaching the colon is fermented to short-chain fatty acid, branched chain fatty acids such as isobutyrate, isovalerate and a range of nitrogenous compounds and ultimately to ammonia. Some of these end products may be toxic to the host, e.g. ammonia, certain amines and phenolic compounds. Increasing the amount of protein in the diet, even easily digestible protein, can increase production of these potentially toxic bacterial metabolites in the colon. Excessive protein fermentation, especially in the distal colon, has been linked with disease states such as colon cancer and ulcerative colitis. Two major factors govern the amount of protein reaching the colon, the total amount of protein in the diet and the digestibility of this protein in the upper gut.
The Maillard reaction occurring between proteins and reducing sugars has been shown to be responsible for production of a large variety of structurally diverse xenobiotic metabolites upon heat treatment of foods. Some of these compounds have been shown to possess antioxidant activities while others are thought to play a role in colon toxicology, through increased colonic protein fermentation, and some such as the advanced glycation end-product (AGE) may also induce inflammation.

Protein digestion and uptake in the gastrointestinal system

Protein digestion

The process of protein digestion starts with maceration of food in the mouth, continues with pepsin digestion in the stomach and ends up with digestion by a mixture of proteolytic enzymes of pankreas (trypsin and chymotrypsin). Protein digestion begins in the stomach, with the action of the hydrochloric acid that is produced there and pepsin. Several factors influence how fast the proteolytic enzymes act on the protein. These factors include the concentration of the enzyme; the amount of protein; the acidity of the food and of the stomach; the temperature of the food; time; and the presence of any digestion inhibitors, such as antacids. Digestion continues in the upper portion of the small intestine under the action of the pancreatic protein enzymes, trypsin and chymotrypsin. The amino acids are absorbed by the blood capillaries of the small intestines, carried through the liver, and then go into the blood of the general circulation.
The major protease of human digestive system is pepsin, an aspartic protease having an optimum of pH around 2. An increase in pH up to 7 leads to significant decrease in catalytic activity of the pepsin, but it can also influence the ionization properties of the substrate, or substrat binding pocket of the enzyme itself. Such alterations as a result have changes in substrate specificity of pepsin, leading to generation of different sets of peptides originating from the substrate. In order to elicit an immune response, a protein needs to be presented to the immune system of the mucosa.

Gut-associated lymphoid tissue

The gastrointestinal system plays a central role in immune system homeostasis. Gut-associated lymphoid tissue (GALT) represents almost 70% of the entire immune system.
The gastrointestinal mucosal immune system has a demanding task in distinguishing between desired and unharmful dietary antigens and intestinal commensal bacterial populations and, on the other hand, possible harmful molecules and pathogenic organisms. The specific environment of the intestine lumen leads to the development of a powerful tolerogenic mechanism known as oral tolerance[12].
A very important portal for presenting antigens in the gut lumen are the organized lymphoid tissue structures known as Peyer's patches (PPs), which are distributed in subepithelia along the intestinal wall, below specialized epithelial cells known as M cells (microfold cells). Antigens entering through the M-cell dependent mechanism are eventually delivered to underlying antigen presenting cells (APCs), dendritic cells (DCs) or macrophages, which migrate into the PPs, priming immune response that way. PPs were believed to be essential for the induction of intestinal immune responses. It is particularly controversial whether PPs are essential for mucosal immune responses and tolerance to soluble antigens. It has been reported that oral tolerance to ovalbumin cannot be induced in mice lacking PPs[13]. How essential PPs are in oral tolerance induction is debatable, however, because tolerance to soluble antigen could be induced in mice lacking PPs [14]. In addition, PP-deficient mice were shown to have

the same frequency of APCs, including DCs and macrophages, in various lymphoid organs after oral administration of a soluble antigen as mice with intact PPs[15].
Another important portal for antigen entry and presentation is via subepithelial dendritic cells. It was found that a much higher proportion of orally administered soluble proteins associates with DC in the lamina propria than in the PPs[16]. In respect of invasive or noninvasive pathogens, DCs are able to induce tolerance or reaction[17].
Antigens can be uptaken and presented to the immune system by intestinal epithelial cells which might act as non-professional antigen-presenting cells and modulate local immune response through the activation of intraepithelial T cells with regulatory functions[18].
It has been shown that hyperpermeability of the gastrointestinal mucosal barrier results in enhanced transport of intact and degraded antigens across the gastrointestinal mucosal barrier, which could favour food protein sensitisation and food allergy in susceptible individuals[19].

Relationship between food allergies and digestion process

Food allergy and food allergens

Food allergy as a common health problem arises from breaking of a natural immunological tolerance to otherwise not harmful food proteins. It is manifested as an abnormal immunological reaction to food proteins, which causes an adverse clinical reaction. The immunological reaction mediated by immunoglobulin class E (IgE) can occur as a consequence of conformational cross-reactivity to respiratory allergens, or following sensitization to the particular protein via the gastrointestinal tract. Almost all known food allergens that sensitize via the gastrointestinal system belong to prolamin and cupin protein superfamilies of allergens. Those are mainly characterized by resistance to heat and digestion. The discrepancy between the vast numbers of proteins we encounter and the limited number of proteins that actually become allergens have led scientists to investigate what unique features make proteins destined to be allergens.
A link between food allergies and digestion process has been observed long time ago[6]. Since then, dietary proteins have been categorized as digestion-resistant class 1 (true allergens triggering direct oral sensitization) or as labile class 2 allergens (nonsensitizing elicitors of a so called oral allergy syndrom). Depeding on age, medicament usage and/or pathological conditions, gastric digestion can become less efficient. More recent evidences have proven the link between impaired gastric digestion and prevalence to food allergies[7].
An efficient gastric digestion may substantially decreases the potential of food proteins to bind IgE, which increases the threshold dose of allergens required to elicit symptoms in patients with food allergy. For some pepsin-labile food allergens, pepsin digestion generates larger peptides, of a length sufficent enough to enable sensitization and subsequent elicitation of food allergy (i.e. major peanut allergen Ara h 2)[20]. Aggregation of proteins and/or digestion generated peptides is other promoting factor in food allergy risk. Many of the true food sensitizers are aggregated proteins or prone to aggregation after common food process-

ing conditions, such as pasteurizaton. Indeed, a recent *in vivo* study showed an increased allergy-promoting effect of pasteurization of whey proteins[21].

In vitro and in vivo allergens digestibility

Based on their potential to trigger specific IgE antibody production food allergens are divided in two classes. The class 1 or complete allergens in addition to their ability to cross-link IgE are the primary source of sensitization. In general, plenty of class 1 allergens are low molecular weight glycoproteins with acidic isoelectric points and most of them are highly abundant in food. These proteins are usually resistant to proteases, heat and denaturants, which preserve them from degradation during food preparation and digestion, thereby enabling direct oral gastrointestinal sensitization[20]. Thus the senzitizing potential of food apparently mirrors situation of gastric proteolysis and digestion assays with simulated gastric fluid have been introduced for characterization of food proteins to imitate the effect of stomach proteolysis on dietary compounds and novel proteins *in vitro*[8]. The class 2 or incomplete food allergens are postulated to lack sensitizing capacity. These proteins have the potential to elicit symptoms only after primary sensitization with cross-reactive inhalative allergens and were therefore termed nonsensitizing elicitors. Examples are protein homologues of Bet v 1, the major birch pollen allergen, which are present in fruits and vegetables. Their susceptibility to pepsin digestion has been demonstrated and might explain why local but not systemic symptoms are frequently triggered after ingestion of Bet v 1 homologues [22]. A similar observation was noted for digestion of kiwi fruit stable versus labile allergens: labile allergens more often bind IgE from patients with localized symptoms (so called oral-allergy syndrome) to kiwi fruit ingestion[23].
Although a correlation between the resistance to pepsin digestion and allergenic potential has been proposed a long time ago[6], the relationship is not absolute and it is still a matter of scientific dispute[24, 25]. In addition, more evidence is building up on protective effects of the food matrix on pepsin and pancreatin digestion of proteins. A special feature of the food matrix is that it may contain substances able to hamper enzymatic digestion by making physical obstacles to enzyme action. Those described so far are lipids i.e., phosphatidylcholine[26], which is secreted by the stomach and also is abundant in milk and polysaccharides i.e. pectin, of fruit matrices[27, 28].

Food processing can tailor food properties and improve food safety

An increase in diet-related disorders and food-related immunopathologies i.e. food allergies especially in industrilized countries has been reportedly linked to the changes in nutritional habits and increased consumption of highly processed food. There is an increased pressure on modern food industry[29] to develop mild technologies that will allow production of foodstuffs of high nutritional value, good funtional characteristics, health-promoting and safe.
Several cross-linking enzymes, most notably microbial transglutaminases, are mild, food-approved biotechnology, used in order to improve food functionality[30]. Various oxidase enzymes from molds and fungi, such as tyrosinase and laccase, have potential as novel highly-

efficient cross-linking tools in food processing[31]. Cross-linking results in increase of the molecular weight of proteins, changes in the three-dimensional structure, charge and surface characteristics of the molecule thus altering the biological properties of food proteins, i.e. susceptibility to proteolysis by digestive enzymes, ligand binding, IgE binding and/or allergenicity and immunogenicity[32, 33].

We have previously shown that cross-linking of beta casein by laccase and tyrosinase reduced IgE binding and basophil activation in allergic patients[33]. Similar results were obtained when beta-lactoglobulin was treated with laccase in the presence of phenolic compound rich sour cherry extract[32]. Treatment of peanut proteins with transglutaminase leads to extensive changes in protein composition of the mixtures, with creation of high molecular mass polymers and oligomers[34], but without significant changes in immunogenic responses in a mice model of peanut allergy (unpublished). Similar results have been obtained by polyphenol oxidase (tyrosinase)/caffeic acid and transglutaminase treatment of peanut allergens[34, 35] and laccase oxidase peanut allergens in the presence of intrinstic polyphenols (unpublished).

Cow's milk allergy (CMA) occurs most often in children under 2 years of age, though it may also occur in adults in a severe form[36]. For patients with persistent bovine milk allergy, the only mean to prevent an allergic reaction is to avoid the offending protein, often very difficult because of the wide application of milk proteins as food ingredients[37].

As cow's milk ranks among the top foods causing childhood allergies, hydrolysate formula have been used as substitutes in infants with cow's milk allergy. However, together with their unpleasant taste, there are some worrying nutritional issues with those formulas, such as growth reduction[38], decrease in plasma protein concentrations and iron-binding capacities, and unbalanced plasma and urinary concentrations of some amino acids[38, 39]. Furthermore, animals given hydrolysated formulas exhibited enhanced proteolytic activities of trypsin and chymotrypsin in the intestine, higher levels of cytochromes in the liver and disordered metabolism of glucocorticoids[40]. Therefore, there is a demand for methods aimed to reduce allergenicity of food proteins being independent of proteolytic enzymes.

Recent research has shown that nonthermal food processing methods, such as high intensity pressure and sonication, or enzymatic cross-linking[41, 42], may also effectively alter digestibility of food proteins[43] and its IgE binding ability[44].

Our recent work investigated effects of a sonication by high intensity ultrasound, on the structure, function and allergenicity of a major whey allergen. Beta-lactoglobulin (BLG) constitutes 50% of whey proteins and it is the major allergen of bovine whey[45]. Its tertiary structure consists of nine antiparallel β-sheet structures forming a so-called β-barrel (or calyx) stabilized by formation of two disulfide bonds[46]. The interior of the calyx contains a hydrophobic pocket, allowing the binding of small hydrophobic molecules such as retinoids, fatty acids, vitamins, and cholesterol[47]. In solution, due to its very compact fold, BLG is highly stable to denaturation and resistant to proteolytic hydrolysis[48].

Our results demonstrated a potential of controlled mild processing conditions in reducing allergenic properties of compact globular proteins due to disruption of conformational IgE binding epitopes in pediatric population of cow's milk allergic patients, while preserving functional characteristics of a protein[49].

Although numerous reports described reduced IgE binding properties of enzymatically processed food allergens, studies dealing with the potential of enzymatically processed foods to induce new sensitization to food allergens and/or influence the capacity of food proteins to induce oral tolerance to processed food allergens are scarce. Highly aggregated, cross-linked, food proteins also may show different behavior in the digestion fluids, providing rational for changed persistance of intact proteins and larger peptides in the gastrointestinal tract comparing to intact food allergens[33]. There is an increased number of reports on the links between processing and modification of food allergens and protein digestibility, intestinal permeability, uptake by immune cells of the mucosa and immunedeviation towards Th2 type allergic hypersensitivity. Pasteurization of milk proteins can influence deviation of the immune response towards Th2 in mice[50]. Nitration of ovalbumin, tested in a mice model of allergy showed an increase in allergenicity of the egg's allergen[51]. Heating of peanut allergens descreases its allergenicy, while allergens isolated from roasted peanut were able to retain its IgE binding capacity[52].

Food matrix may influence the liberation of peptides and other bioactives

Components of food matrix can interact noncovalentlly with food allergens giving both soluble and insoluble complexes. Complexation with components of food matrix can lower the of level soluble allergens thus reducing their allergenic properties and/or influencing digestion in the gastrointestinal system by hindering cutting sites from the action of digestive enzymes and/or directly inhibiting digestive enzymes.

Protein rich food matrix

A recent studies examined the effects of protein-rich food matrix on gastrointestinal proteolysis, epithelial transport and *in vivo* absorption of class 2 (Mal d 1, a major apple allergen) and class 1 food allergens (Bos d 5, a major cow's milk allergen and Cor a 8, a major hazelnut allergen)[53]. The presence of hazelnut and peanut extracts, which served as protein-rich model food matrices, delayed gastrointestinal degradation and reduced epithelial transport rates of all allergens through CaCo-2 monolayers. IgE-reactive allergens were assessed at different time points in sera from rats fed with all three allergens with or without hazelnut extract. The levels of all allergens peaked 2 h after animals were fed without matrix and increased over 8 h after feeding. A protein-rich food matrix thus delays gastrointestinal digestion and epithelial transport of food allergens and thereby may affect their sensitizing capacity and clinical symptoms.

Acidic polysaccharides

Mouecoucou et al.[54] examined the influence of polysaccharides, i.e., gum arabic, low methylated pectin (LMP) and xylan, on the *in vitro* hydrolysis of peanut protein isolate (PPI) and the *in vitro* allergenicity of the digestion products. PPI was hydrolyzed *in vitro* by pepsin, followed by a trypsin/ chymotrypsin (T/C) mixture in dialysis bags. Hydrolysis by all of the digestive enzymes showed retention of some proteins in the dialysis bags in the presence

of gum arabic and xylan. The retentates were recognized by IgG and IgE, but IgE binding of retentate containing xylan was reduced. The immunoreactivity of hydrolysis products in dialysates was considerably reduced by polysaccharides. Our previous study demonstrated that addition of apple fruit pectin (1.5% and 3%) to the purified kiwi allergen Act c 2 was able to protect it from pepsin digestion in vitro . Similarly, *in vitro* digestion of actinidin, another major allergen of kiwi fruit was hapmered by pectin in both gastric and duodenal fluids[28]. *In vivo* experiments on healthy non-atopic volunteers have shown that 1 h after ingestion of kiwi fruit in gastric content intact Act c 2 was still present[27]. Similarly, after *in vivo* digestion of Act c 2 in rats in the presence of apple pectin, both specific and total pepsin activity declined and thus protected 23% of the ingested allergen from digestion for 90 minutes[55]. Protective effect of various fruit pectins on the intestinal digestion of allergens correlleted well with their ability to form hydrogel network and provide a physical obstacle to the pepsin action.

These results show that although presence of polysaccharides can be effective in masking of IgE epitopes, thus reducing allergenicity, it also reduces allergen digestibility, enabling higher dosages of the intact allergen to reach the immune system.

Dietary polyphenols of a food matrix interact with proteins and exert various health effects

Polyphenols, including flavonoids, phenolic acids, proanthocyanidins and resveratrol, are a large and heterogeneous group of phytochemicals in plant-based foods, such as tea, coffee, wine, cocoa, cereal grains, soy, fruits and berries. Naturally occurring polyphenols are potent antioxidants and exert many beneficial effects on human health which have led to their recognition as potential nutraceuticals. In solution, polyphenols can form non-covalent complexes with globular proteins and such interaction may result in complexation[56, 57], stabilization of protein structure[58], protein unfolding and precipitation[59-61]. The strength of interactions depends on the size of polyphenols, polyphenols structure and amino-acid sequence of proteins[62].

The effect of some phenolic compounds, resveratrol, catechin, epigallocatechin-3-gallate, quercetin and phenolics-rich beverages (red wine and green tea) on pepsin activity has been described previously. The tested polyphenols and beverages increased the initial velocity of the reaction, affecting the Vmax of pepsin on denaturated haemoglobin as a substrate, and the activating effect is concentration dependent[63]. On the contrary, many *in vitro* and *in vivo* studies showed antinutritive properties of polyphenols, especially tannins[64].

The ability of polyphenolic compounds to form insoluble complexes with proteins has long been associated with the observed reduction in nutritive value resulting from their inclusion in animal diets. In addition, such complexation may reduce IgE binding of allergens due to irreversible precipitation, as recently shown for complexes of peanut allergens and phenolic acids[65].

We have recently shown that enzymatic processing by a phenol oxidase, laccase, and usage of fruit phenolic extracts as mediators of enzymatic reactions may enable partial cross-

linking of beta-lactoglobulin (BLG)[32, 66]. The cherry phenolics extract facilitated pepsin and trypsin digestion of the remaining monomeric BLG, while apple polyphenols protected cross-linked proteins from both pepsin and pankreatin digestion, presumably by forming a firm physical network[66].

In a recent study, we have examined simulated gastric digestion of several food allergens (BLG and alpha-lactalbumin from cow's milk, peanut extract proteins in the presence of catechin-enriched food supplements of green tea (GTC), polymerized catechins and phenol oxidase processed mixtures of food allergens and green tea catechins. GTC facilitated pepsin digestion of all tested allergens in a dose-dependent manner. Polymerized GTC affected adversely protein digestion of BLG and alpha-lactoalbumine, but not protein digestion of peanut extract proteins. Protecting effect of polyphenols correlated well with the ability of proteins to form insoluble complexes with oxidized catechins[67]. The main biological impact of anti-nutritive feature of polyphenols in the context of food proteins digestion may be reduced bioavailability of peptides in the gastrointestinal system.

Our recent work also investigated the impact of different polyphenols of food baverages on pepsin and pankreatin digestion of BLG in the conditions that were subomptimal for protein precipitation, and gave an insight into the mechanism of protein protection from enzymatic digestion in relation to the binding affinity of polyphenols to the protein. The results of our work show that dietary polyphenols act inhibitory at all stages of a globular food protein digestion thoughout the gastrointestinal tract and that the most effective protection was observed with coffee polyphenols due to the highest binding affinity and stabilizing effect on the protein structure. The exerted effect is related to the non-covalent interactions in solution and occurs also in the absence of protein precipitation. We have demonstrated that protective action of polyphenols also involve non-covalent interactions in solution which occur at the range of pH values that occur in the digestive tract and provide protection from digestive enzymes. In addition, dietary polyphenols are able to stabilize protein secondary structure at the extremely acidic pH of the stomach, thus prolonging the half-life of the protein in *in vitro* digestibility assay[68].

Besides effects on digestion, complexation of polyphenols and proteins can affect antioxidant activity of polyphenols[69]. Even though the effect of complexation can be reduction of antioxidative activities of both protein and polyphenol, due to prolonged life of polyphenol in a complex, the effect of complexation may be beneficial for the overall antioxidative activity of the polyphenol.

Concluding remarks

Digestion is the key step for delivering bioactives to the body and a huge effort has to be made for better understanding the mechanism of food digestion and the relationships with human health. Complex food matrices cannot be adequately described as mixtures of macronutrients (proteins, lipids, sugars). The way the different components are associated to form the food matrix can significantly modify the effect of food on human health. Understanding how food structures are modified during processing, and disintegrated during digestion, will

allow rational design of healthier foods and hence will maximise beneficial health effects of our diet. It will give new inputs to the food industry for designing innovative functional food products.

Acknowledgements

The authors acknowledge support of the GA No. 172024 of the Ministry of Education, Science and Technological Development of the Republic of Serbia and FP7 RegPot project FCUB ERA GA No. 256716. The EC does not share responsibility for the content of the article. The authors are participants in the COST Action FA1005 Infogest on food digestion.

References

1. Walther B, Sieber R, (2011). Bioactive proteins and peptides in foods. *Int J Vitam Nutr Res*, **81**(2-3):181-192.
2. Moller NP, *et al.,* (2008). Bioactive peptides and proteins from foods: indication for health effects. *Eur J Nutr*, **47**(4):171-182.
3. Danesi F, *et al.,* (2010). Food-derived bioactives as potential regulators of the IL-12/IL-23 pathway implicated in inflammatory bowel diseases. *Mutat Res*, **690**(1-2):139-144.
4. Ricci-Cabello I, Herrera MO, Artacho R, (2012). Possible role of milk-derived bioactive peptides in the treatment and prevention of metabolic syndrome. *Nutr Rev*, **70**(4):241-255.
5. Phelan M, Kerins D, (2011). The potential role of milk-derived peptides in cardiovascular disease. *Food Funct*, **2**(3-4):153-167.
6. Astwood JD, Leach JN, Fuchs RL, (1996). Stability of food allergens to digestion in vitro. *Nat Biotechnol*, **14**(10):1269-1273.
7. Untersmayr E, Jensen-Jarolim E, (2008). The role of protein digestibility and antacids on food allergy outcomes. *J Allergy Clin Immunol*, **121**(6):1301-1308; quiz 1309-1310.
8. Wickham M, Faulks R, Mills C, (2009). In vitro digestion methods for assessing the effect of food structure on allergen breakdown. *Mol Nutr Food Res*, **53**(8):952-958.
9. Ladics GS, (2008). Current codex guidelines for assessment of potential protein allergenicity. *Food Chem Toxicol*, **46 Suppl 10**:S20-23.
10. Herman RA, Storer NP, Gao Y, (2006). Digestion assays in allergenicity assessment of transgenic proteins. *Environ Health Perspect*, **114**(8):1154-1157.
11. Compare D, Nardone G, (2011). Contribution of gut microbiota to colonic and extracolonic cancer development. *Dig Dis*, **29**(6):554-561.
12. Mayer L, Shao L, (2004). Therapeutic potential of oral tolerance. *Nature Reviews: Immunology*, **4**(6):407-419.
13. Fujihashi K, *et al.,* (2001). Peyer's patches are required for oral tolerance to proteins. *Proc Nat Acad Sci U S A*, **98**(6):3310-3315.
14. Spahn TW, *et al.,* (2001). Induction of oral tolerance to cellular immune responses in the absence of Peyer's patches. *Europ J Immun*, **31**(4):1278-1287.
15. Kunkel D, *et al.,* (2003). Visualization of peptide presentation following oral application of antigen in normal and Peyer's patches-deficient mice. *Europ J Immun*, **33**(5):1292-1301.
16. Mowat AM, (2005). Dendritic cells and immune responses to orally administered antigens. *Vaccine*, **23**(15):1797-1799.

17. Bilsborough J, Viney JL, (2004). Gastrointestinal dendritic cells play a role in immunity, tolerance, and disease. *Gastroenterology*, **127**(1):300-309.
18. Allez M, Brimnes J, Dotan I, Mayer L: Expansion of CD8+ T cells with regulatory function after interaction with intestinal epithelial cells. *Gastroenterology* 2002, **123**(5):1516-1526.
19. Farhadi A, Banan A, Fields J, Keshavarzian A: Intestinal barrier: an interface between health and disease. *J Gastroenterol Hepatol* 2003, **18**(5):479-497.
20. Cirkovic Velickovic T, Radosavljevic J, Stanic D, (2009). Digestibility of food allergens. *CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources* 9, **4**(6).
21. Roth-Walter F, *et al.,* (2008).Pasteurization of milk proteins promotes allergic sensitization by enhancing uptake through Peyer's patches. *Allergy*, **63**(7):882-890.
22. Vieths S, Scheurer S, Ballmer-Weber B, (2002). Current understanding of cross-reactivity of food allergens and pollen. *Ann N Y Acad Sci*, **964**:47-68.
23. Lucas JS, *et al.,* (2008). The effect of digestion and pH on the allergenicity of kiwifruit proteins. *Pediatr Allergy Immunol*, **19**(5):392-398.
24. Fu TJ, Abbott UR, Hatzos C, (2002). Digestibility of food allergens and nonallergenic proteins in simulated gastric fluid and simulated intestinal fluid-a comparative study. *J Agric Food Chem*, **50**(24):7154-7160.
25. Fu TJ (2002). Digestion stability as a criterion for protein allergenicity assessment. *Ann N Y Acad Sci*, **964**:99-110.
26. Moreno FJ, Mackie AR, Mills EN, (2005). Phospholipid interactions protect the milk allergen alpha-lactalbumin from proteolysis during in vitro digestion. *J Agric Food Chem*, **53**(25):9810-9816.
27. Polovic N, *et al.,* (2007). A matrix effect in pectin-rich fruits hampers digestion of allergen by pepsin in vivo and in vitro. *Clin Exp Allergy*, **37**(5):764-771.
28. Polovic ND, *et al.,* (2009) Acid-formed pectin gel delays major incomplete kiwi fruit allergen Act c 1 proteolysis in in vitro gastrointestinal digestion. *J Sci Food Agric*, **89**(1):8-14.
29. Buchert J, *et al.,* (2010). Crosslinking Food Proteins for Improved Functionality. In: *Ann Rev Food Sci Technol,* **1:** 113-138.
30. Aboumahmoud R, Savello P, (1990). Crosslinking of whey protein by transglutaminase. J Dairy Sci, 73(2):256-263.
31. Selinheimo E, *et al.,* (2008). Formation of protein-oligosaccharide conjugates by laccase and tyrosinase. *J Agric Food Chem*, **56**(9):3118-3128.
32. Tantoush Z, *et al.,* (2011). Digestibility and allergenicity of beta-lactoglobulin following laccase-mediated cross-linking in the presence of sour cherry phenolics. *Food Chemistry*, **125**(1):84-91.
33. Partschefeld C, *et al.,* (2007).Crosslinking of casein by microbial transglutaminase and its resulting influence on the stability of micelle structure. *Biotech J*, **2**(4):456-461.
34. Clare DA, Gharst G, Sanders TH, (2007). Transglutaminase polymerization of peanut proteins. *J Agric Food Chem*, **55**(2):432-438.
35. Chung SY, Kato Y, Champagne ET, (2005). Polyphenol oxidase/caffeic acid may reduce the allergenic properties of peanut allergens. *J Sci Food Agric*, **85**(15):2631-2637.
36. Lam HY, *et al.,* (2008). Cow's milk allergy in adults is rare but severe: both casein and whey proteins are involved. *Clin Exp Allergy* 8, **38**:995-1002.
37. Boyano-Martínez T, *et al.,* (2009). Accidental allergic reactions in children allergic to cow's milk proteins. *J Allergy Clin Immun*, **123**(4):883-888.

38. Hernell O, Lonnerdal B, (2003). Nutritional evaluation of protein hydrolysate formulas in healthy term infants: plasma amino acids, hematology, and trace elements. *Am J Clin Nutr*, **78**(2):296-301.
39. Maggio L, *et al.,* (2005). Higher urinary excretion of essential amino acids in preterm infants fed protein hydrolysates. *Acta Paediatr*, **94**(1):75-84.
40. Malikova NA, *et al.,* (1993). IN: Effect of milk proteins and their enzymatic hydrolyzates on non-specific resistance of some systems to food allergens. *Vopr Pitan* (1):26-30.
41. Ross AIV, *et al.,* (2003). Combining nonthermal technologies to control foodborne microorganisms. *Int J Food Microbiol*, **89**(2-3):125-138.
42. Stanic D, *et al.,* (2010). Digestibility and allergenicity assessment of enzymatically crosslinked beta-casein. *Mol Nutr Food Res* 2010, **54**(9):1273-1284.
43. Yu H-L, *et al.,* (2011). Effects of different processing methods on digestibility of Scylla paramamosain allergen (tropomyosin). *Food Chem Toxicol*, **49**(4):791-798.
44. Husband FA, *et al.,* (2011). High-pressure treatment reduces the immunoreactivity of the major allergens in apple and celeriac. *Mol Nutr Food Res*, **55**(7):1087-1095.
45. Shek LP, *et al.,* (2005). Humoral and cellular responses to cow milk proteins in patients with milk-induced IgE-mediated and non-IgE-mediated disorders. *Allergy*, **60**(7):912-919.
46. Fogolari F, *et al.,* (1998). Monomeric bovine beta-lactoglobulin adopts a beta-barrel fold at pH 2. *Febs Letters*, **436**(2):149-154.
47. Kontopidis G, Holt C, Sawyer L, (2002). The ligand-binding site of bovine beta-lactoglobulin: Evidence for a function? *J Mol Biol*, **318**(4):1043-1055.
48. Breiteneder H, Mills ENC, (2005). Molecular properties of food allergens. *J Allergy Clin Immun*, **115**(1):14-23.
49. Stanic-Vucinic D, *et al.,* (2012). Structural changes and allergenic properties of ß-lactoglobulin upon exposure to high-intensity ultrasound. *Mol Nutr Food Res*, **in press**.
50. Roth-Walter F, *et al.,* (2008). Pasteurization of milk proteins promotes allergic sensitization by enhancing uptake through Peyer's patches. *Allergy*, **63**(7):882-890.
51. Untersmayr E, *et al.,* (2010). Nitration of the egg-allergen ovalbumin enhances protein allergenicity but reduces the risk for oral sensitization in a murine model of food allergy. *PLoS One*, **5**(12):e14210.
52. Vissers YM, *et al.,* (2011). Effect of Heating and Glycation on the Allergenicity of 2S Albumins (Ara h 2/6) from Peanut. *PLoS One*, **6**(8).
53. Schulten V, *et al.,* (2011). A food matrix reduces digestion and absorption of food allergens in vivo. *Mol Nutr Food Res*, **55**(10):1484-1491.
54. Mouecoucou J, *et al.,* (2004). In vitro allergenicity of peanut after hydrolysis in the presence of polysaccharides. *Clin Exp Allergy*, **34**(9):1429-1437.
55. Polovic N, *et al.,* (2012). In vivo digestion of a thaumatin-like kiwifruit protein in rats. *Food Digestion*, **1**(1-2):5-13.
56. Chaudhuri S, Chakraborty S, Sengupta PK, (2011). Probing the interactions of hemoglobin with antioxidant flavonoids via fluorescence spectroscopy and molecular modeling studies. *Biophys Chem*, **154**(1):26-34.
57. Li SA, *et al.,* (2010). Comparative studies on the interaction of caffeic acid, chlorogenic acid and ferulic acid with bovine serum albumin. *Spectrochim Acta A Mol Biomol Spectrosc*, **77**(3):680-686.

58. Kanakis CD, *et al.,* (2011). Milk beta-lactoglobulin complexes with tea polyphenols. *Food Chemistry*, **127**(3):1046-1055.
59. Ma J, *et al.,* (2011). Interactions of Flavonoids with Biomacromolecules. *Current Organic Chemistry*, **15**(15):2627-2640.
60. Papadopoulou A, Frazier RA, (2004). Characterization of protein-polyphenol interactions. *Trends Food Sci Technol*, **15**(3-4):186-190.
61. Siebert KJ, Troukhanova NV, Lynn PY, (1996). Nature of polyphenol-protein interactions. *J Agric Food Chem*, **44**(1):80-85.
62. Frazier RA, *et al.,* (2010). Interactions of tea tannins and condensed tannins with proteins. *J Pharm Biomed Analysis*, **51**(2):490-495.
63. Tagliazucchi D, Verzelloni E, Conte A, (2005). Effect of some phenolic compounds and beverages on pepsin activity during simulated gastric digestion. *J Agric Food Chem*, **53**(22):8706-8713.
64. Bravo L (1998). Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. *Nutr Rev*, **56**(11):317-333.
65. Chung SY, Champagne ET, (2009). Reducing the allergenic capacity of peanut extracts and liquid peanut butter by phenolic compounds. *Food Chem*, **115**(4):1345-1349.
66. Tantoush Z, *et al.,* (2011). Digestibility of beta-lactoglobulin following cross-linking by Trametes versicolor laccase and apple phenolics. *J Serb Chem Soc*, **100**(1):80-90.
67. Tantoush Z, *et al.,* (2012). Green tea catechins of food, supplements facilitate pepsin digestion of major food allergens, but hampers their digestion if oxidized by phenol oxidase. *J Func Foods*, **4**(3):650-660.
68. Stojadinovic M, *et al.,* (2012). Binding affinity between dietary polyphenols and <beta>-lactoglobulin negatively correlates with the protein susceptibility to digestion and total antioxidant activity of formed complexes. *Food Chem*, **in press**.
69. Arts MJTJ, *et al.,* (2002). Interactions between Flavonoids and Proteins: Effect on the Total Antioxidant Capacity. *J Agric Food Chem*, **50**(5):1184-1187.

Topology of proteasomal core particle of *Haloferax volcanii* by chemical cross-linking, mass spectrometry and bioinformatics

Ivanka Karadžić[1*], Julie Maupin-Furlow[2], David Goodlett[3]

[*] [1]*Department of Chemistry, School of Medicine, University of Belgrade, Belgrade, Serbia*
[2]*Department of Microbiology and Cell Science, University of Florida, Gainesville, Florida, USA*
[3]*Department of Medicinal Chemistry, School of Pharmacy, University of Washington, Seattle, Washington, USA*
e-mail: ikaradzic@med.bg.ac.rs

Proteasomal 20S core particles (CPs) from the haloarchaeon *Haloferax volcanii* are cylindrial barrel-like structures of four-stacked homoheptameric rings of α- and β-type subunits organized in $\alpha_7\beta_7\beta_7\alpha_7$ stoichiometry. Chemically cross-linked peptides of the *H. volcanii* CPs were analyzed by high-performance mass spectrometry and an open modification search strategy. Distance constraints obtained by chemical cross-linking mass spectrometry (CXMS), together with the available structural data of non-halophilic CPs, facilitated the selection of accurate models of *H. volcanii* proteasomal CPs composed of α1 and β-homoheptameric rings from among several different possible PDB structures.

Introduction

Significant progress has been made toward determination of spatial and topological organization of protein and protein complexes by traditional methods such as: nuclear magnetic resonance (NMR) spectroscopy and X-ray crystallography, which provide detailed information on the structure of highly purified proteins, but have limitation when analyzing protein complexes[1,2]. In the post genomic era, large-scale analysis of protein structure by sensitive, high-throughput techniques, such as mass spectrometry (MS), play a significant role. Among the few MS techniques used for structural analysis of proteins, chemical cross-linking coupled with mass spectrometry (CXMS) has emerged as a method that can yield site-specific low resolution structure information on the distance constraints with sample quantities in two to three orders of magnitude less than required for X-ray and NMR and with rapid experimental time[1].

Chemical Cross-Linking Coupled with Mass Spectrometry (CXMS)

The general principle of CXMS is the covalent capture of juxtaposed amino acids using a variety of cross-linking reagents. CXMS can provide intra- and inter-molecular distance constraints that can be used to resolve protein folding in monomeric protein subunits and interactions at molecular interfaces in protein complexes. A CXMS analytical approach has been recently reported that is based on high performance MS to generate tandem mass spectra and the open modification, targeted de novo search strategy to interpret the data[3]. The designed method can be used to map both chemically and naturally occurring cross-links in proteins. Each peptide in the pair of cross-linked peptides is considered to be post-translationally modified by the other peptide in the pair such that each has an unknown mass at an unknown amino acid. This approach allows the use of any available open modification search engine for data analysis. To facilitate identification of cross-linked peptides and to make CXMS data analysis more generally applicable across laboratories, a database processing tool referred to as xComb has been developed[4]. xComb can be used with any standard database search engine and is publicly available.

Cross-Linking Reagents

A large and increasing set of chemical cross-linking reagents has been investigated for use as a molecular ruler to provide information on distances between cross-linked amino acid residues that are relevant to both the tertiary and quaternary structure of proteins. Aside from the large number of described and commercially available cross-linkers that are utilized, only a few organic reactions based on nucleophilicity of the functional groups of proteins are used to attach the cross-linkers[5]. Cross-linking reagents targeting amino groups in proteins are the most common reagents. Based on the reaction of acylation of ε-amino group of lysine, a list of homobifunctional and heterobifunctional cross-linkers containing N-hydroxysuccinimide esters has been synthesized and used in CXMS protocols[5]. Homobifunctional N-hydroxysuccinimide (NHS) esters primarily target the ε-amino group of lysine residues of proteins and can differ in the length of the spacer arm from 6.4 to 11.4 Å. Although the most commonly used NHS esters are often described as reactive exclusively towards primary amines, side reactions with other amino acid residues have also been reported. In particular, NHS reactivity with serine, threonine and tyrosine residues is significant but highly dependent on the pH and on adjacent amino acids within the protein[6]. In contrast to most bifunctional reagents such as NHS, which introduce a bridge between cross-linked residues, the zero length carbodiimide cross-linkers mediate creation of a covalent bond between carboxylate and an amine group without an intervening linker, allowing a direct evaluation of contact interactions between protein surfaces[5].

CXMS and Comparative Modeling

Distance determinations obtained by the CXMS technique can lead to important advances in mapping the protein topography in low resolution structures refined by computational

methods. *Ab initio* and comparative modeling of protein complexes result in a number of possible structures. Although the number of actual proteins is vast, most proteins belong to a limited set of tertiary structural motifs[7,8]. Thus, comparative protein modeling, which uses previously solved structures/templates as starting point, can be very effective in providing a preliminary estimate of protein structure. Distance constraints generated by CXMS can then facilitate the evaluation and/or construction of the modeled protein structure. Even a small number of intermolecular cross-link constraints are sufficient to validate the topology prediction of a protein complex[9,10,11]. Thus, acquisition of cross-linking distance constraints of protein assemblies is a realistic approach to use on a routine basis to improve modeling accuracies[9]. The gap between CXMS and protein structure modeling has been addressed by a new software platform called MSX-3D, which facilitates validation of a theoretical models based on CXMS data[12].

20S Proteolytic Core Particles (CPs) from the Haloarchaeon *Haloferax volcanii*

Archaea are one of three major evolutionary lineages of life. Recent advances in the genome sequencing of halophilic archaea (or haloarchaea) have made this group of microbes ideal for providing insight into cell physiology[13]. Haloarchaea have developed into model organisms that are used to study many biological processes, including the function of the ubiquitin-proteasome system[25]. Proteasomes from archaea are highly related to those of eukaryotes. *Haloferax volcanii* is the only archaeon that has been demonstrated to synthesize three different proteasomal proteins: α1, α2 and β [encoded by *psmA* (HVO_1091), *psmC* (HVO_2923) and *psmB* (HVO_1562), respectively] that are classified in the 20S proteasome superfamily[14].

Proteasomes are self-compartmentalized nanomachines, composed of triple A-ATPases (Rpts) and 20S core particles (CPs) that are important in proteolysis and often essential for growth[13]. The proteasomal CP is a cylindrical complex of four-stacked heptameric rings of α- and β-type subunits organized in $\alpha_7\beta_7\beta_7\alpha_7$ stoichiometry. The α-type subunits form the outer rings and the β-type subunits form the two inner rings that harbor the proteolytic active sites[15, 16]. As *H. volcanii* synthesizes two different α subunits (α1 and α2), this organism has the potential to make three different CPs: two symmetric (α1ββα1, α2ββα2) and one asymmetric (α1ββα2)[15]. Since the α1 and α2 proteins share only 55.5% identity, significant structural differences in the homoheptameric rings formed by α1 and α2 are predicted[15].

Structural characterization of eukaryotic proteasomes by CXMS has been recently published[16, 17]. Although electron micrographs of the proteasomal CPs from *H. volcanii* reveal a four-stacked ring structure with a central channel[14], the structural details for these protein complexes are limited. PDB entries of archaeal proteasomes deposited to date in RCSB protein data bank are: 1j2q of *Archaeoglobus fulgidus*[23], 3h4p of *Methanocaldococcus jannaschii*[26], and 1pma of *Thermoplasma acidophilum*[27]. All deposited archaeal proteasomes are non-halophilic.

Halophilic CP nanomachines by CXMS

Halophilic proteins are quite specific with regard to amino acid composition often having an extremely high content of acidic amino acids and unusual abundance of surface exposed acidic residues[18]. With regard to CXMS investigation of *H. volcanii* CPs, several points must be emphasized. First, haloarchaeal proteins are typically halophilic (salt-loving) comprising a high content of acidic residues that are often surface exposed and a low number of basic amino acid residues (*e.g.*, lysine, a target of many cross-linkers). In addition, the *H. volcanii* CPs do not contain any cysteine residues. All of these factors limit and complicate the investigation of the halophilic CPs by CXMS, since lysine and cysteine residues are often targets of commercial cross-linkers. Furthermore, only a few crystal structures of haloarchaeal proteins and proteasomal CPs (with the latter derived only from non-halophilic archaea or eukarya) have been resolved to date. In spite of these limitations regarding halophilic proteasomes, CXMS coupled with protein modeling, could emerge as a powerful approach to predict the structure of these unusual, acidic proteins.

Taking into account advantages of CXMS to elucidate tertiary and quaternary structure of proteins, a symmetric $\alpha1\beta\beta\alpha1$ core particle of *H. volcanii* proteasomes was studied using two commercial chemical cross-linkers. Zero length EDC and homobifunctional amine-specific BS2G were used as cross-linkers to determine the juxtaposed amino acids in the CP and to validate the theoretical 3D protein structures predicted by comparative modeling[24].

Identification of cross-linked peptide

Tandem mass spectra (MS/MS) were analyzed to confirm the identity and to evaluate the type of cross-linked peptides. To identify cross-linked peptides, MS data were acquired and analyzed using the open modification search strategy reported by Singh *et al.*[3]. Briefly, all data were generated at high mass accuracy on an LTQ-Orbitrap mass spectrometer (Thermo Fisher, USA) using data dependent acquisition of tandem mass spectra by collision induced dissociation (CID) of ions $\geq (M+4H)^{4+}$. The resulting database was searched and refined with respect to the used cross-linkers by xComb and uploaded in Phenyx. Tandem mass spectra of cross-linked peptides were annotated using MS2Assign[19]. Strict criterions were applied on MS2 analysis: 1. Fragment ions from both cross-linked peptides had to be assigned; 2. Cross-linked amino acid blocks peptide cleavage by proteases, so peptides that contain one missed cleavage were considered; 3. Fragment ions containing cross-linked sequences of both peptides had to be assigned; 4. High mass accuracy was prioritized. According to the classification of cross-linking products three types of single modification were considered: type 0 was defined as dead-end, type 1 was defined as intrapeptide and type 2 was defined as interpeptide with the two peptides denoted as α and β chains, where α is the longer of the two peptides[19].

Protein structure validation

Theoretically calculated models of α1 and β protein subunits were obtained from the publicly available MODBASE (http://modbase.compbio.ucsf.edu/modbase-cgi/index.cgi. Structures designated as α1 (Model ID: 4de8eea6b134a909401c34d144ce816c) and β (Model ID: b0e4c2ed79c3a4a1971f2bb8e71bd170) were used. To analyze 3D protein models using mass spectrometry data, MSX-3D, version 3.4.23 (http://proteomics-pbil.ibcp.fr/cgi-bin/msXsetup.pl) was used[12]. Pairwise comparison of protein structures was established using DaliLite workbench software (http://www.ebi.ac.uk/Tools/dalilite/index.htmL)[20]. The coordinates of heptameric complexes of α1 and β were obtained by DaliLite software using PDB entries 1j2p, 1ryp, and 1fnt (for α1) and 1ryp, 1j2q, and 1fnt (for β). Coordinates of the PDB structures with high Z scores and a strong match to α1 and β subunits were used to assemble proteins into heptameric ring structures of $\alpha 1_7$ and β_7 and double heptameric rings of $\beta_7\beta_7$ by Vega ZZ molecular modeling software[21].

Identification of juxtaposed amino acids by CXMS and protein structure validation

To analyze the symmetric α1ββα1 CP subtypes of *H. volcanii* by CXMS the following strategy was used: 1. CP complex was treated with the homobifunctional lysine-reactive cross-linker BS2G and the zero length EDC cross-linkers; 2. Tandem mass spectra of the cross-linked CPs were analyzed using the open modification search strategy, which is essentially a targeted de novo search, to confirm the identity and to evaluate the type of cross-linkages[3]; 3. distance constraints obtained by CXMS were used for validation of theoretical models of α1 and β subunits by MSX-3D[12]. In addition to MSX-3D, VEGA ZZ program was used to display, analyze, and manage the three dimensional (3D) structure of the protein complexes[21] and to validate 3D structures of protein complexes of α1, and β subunits obtained by homology modeling according to the PDB templates: 1j2p (α-ring of the proteasome from *Archaeoglobus fulgidus*), 1ryp (20S proteasome from the yeast *Saccharomyces cerevisiae*), and 1fnt (20S proteasome from the yeast *Saccharomyces cerevisiae* in complex with the proteasomes activator PA26 from *Trypanosome brucei*). A stoichiometry of seven α1 and β subunits assembled in homoheptameric rings was considered, and the coordinates of this α- and β- ring were obtained by DaliLite software[20].

Several α1-specific linkages were detected by tandem MS after treatment of the α1ββα1 CP subtype with the homobifunctional lysine-reactive BS2G (α1 K54-K68 and K44-K47 cross-linkage) and zero-length EDC (α1 K54-E67 and K163-E62 cross-linkages). Of all the lysine residues within α1, only the intramolecular distance between K54 and K68 (9.4 Å) was found (Fig. 1d) to accomodate the length of the BS2G cross-linker spacer arm (7.7 Å) and generate an α1-interpeptide linkage. Consistent with this result, the span between reactive side chain atoms is often slightly greater than the length of the cross-linker used to connect them[22].

In accordance with the close proximity of the α1 lysine residues K54 and K68 in space, the α1 peptides in the region cross-linked by BS2G were also cross-linked with zero-length EDC (K54 and E67 were linked within α1-peptides 44-TPEGVVLAADKR-55 and 58-SPLMEPTSVEK-68), with intramolecular distance of 6.9 Å between K54 and E67. The α1 peptide 58-SPLMEPTSVEK-69 was also found connected to a peptide in the central region of α1, 150-LYETDPSGTPYEWKAVSIG-171, with fragment ions consistent with a linkage between K163 and either E62 or E67. While an intramolecular distance of greater than 30 Å between K163 and E62/67 was found, intermolecular cross-links between two α1 subunits were measured to be: 4.8 Å, 6.7 Å, and 9.7 Å, for models based on PDB coordinates of 1j2p, 1fnt, and 1ryp, respectively. The EDC cross-link identified in the heptameric α1-ring between K163 and E62 was optimally modeled using 1j2p (Fig. 1a) as a template and was estimated to have a length of 4.8 Å (Fig. 1b).

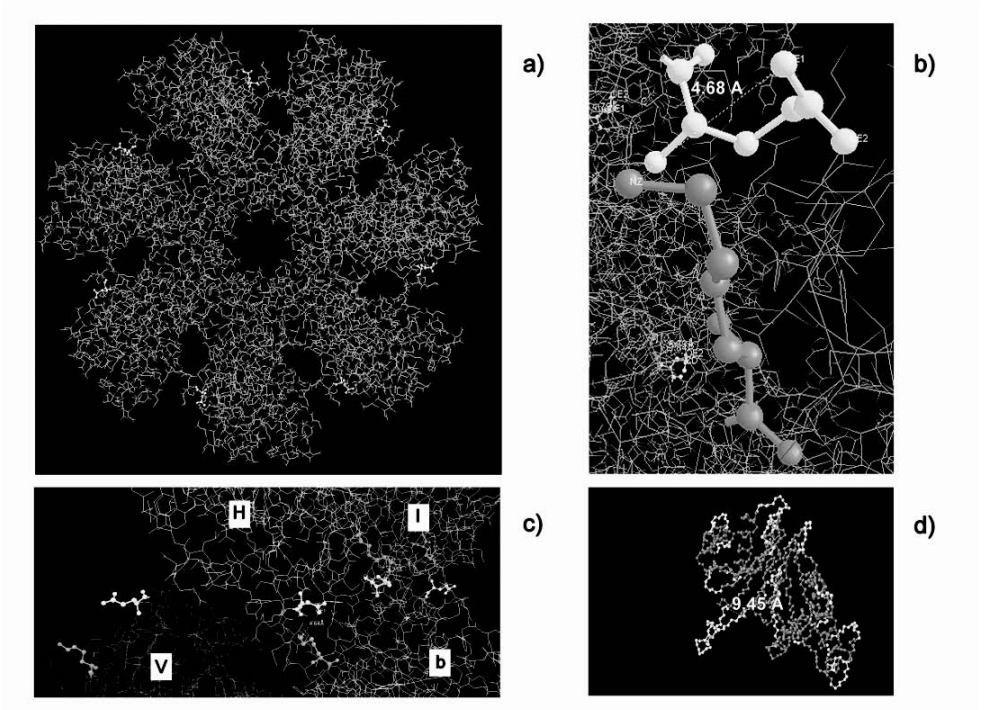


Figure 1. Structural models of the $\alpha 1_7$-ring and $\beta_7\beta_7$-rings of *H. volcanii* 20S CPs. a) Heptameric $\alpha 1_7$-ring modeled according 1j2p PDB template, b) distances between the NZ of K163 and OE1 of E-62 from the two α1 subunits estimated at 4.7 Å, c) the model of the $\beta_7\beta_7$-rings was assigned according to the PDB template 1fnt, as H, I, V, and b. The measured distance between K-78 from the b chain and E-212 from the H chain was 4.64 Å, d) MSX-3D predicts that lysine K54 from TPEGVVLAADKRSR cross-linked with lysine K68 from SPLMEPTSVEKIHK is at a distance of 9.45 Å in the model structure of the α1 subunit.

With BS2G as a cross-linker, four dead-end modifications were detected in α1 tryptic peptides, suggesting that the highly-reactive lysine residues (K68, K71, K54 and K163) are on the surface of α1.
An intrapeptide BS2G cross-linkage was detected between residues K44 and K47 of the β peptide 38-ADELGDKETKTGTTTVGIKTEEGVVLATDMRASMGYMVSSK-78. The distance between β K44 and K47 using BS2G, was measured to be a short intramolecular distance of 6.1 Å by MSX-3D. Between β K78 from 69-ASMGYMVSSKDVQK-82 and β E212 from 209-SAVER-213, cross-linked with EDC the large intramolecular distance of >30 Å was measured. However, the intermolecular distance between the β K78-E212 residues within *two* rings β7β7, was measured to be 4.6 Å in 1fnt (Fig. 1c) and 22.1 Å for 1ryp-based structure. In contrast, distances of 29.4 Å, 16.4 Å, and 26.5 Å were measured between the same residues within *one* ring for 1fnt-, 1j2q-, and 1ryp- based structures, respectively. Thus, the model based on 1fnt appears more accurate for the packing of the $\beta_7\beta_7$-rings than the models based on 1ryp and 1j2q.

Conclusion

The α1ββα1 CP from *H. volcanii* are presumed to be associated as four-stacked heptameric rings in an $\alpha_7\beta_7\beta_7\alpha_7$ symmetry based on analogy to non-halophilic archaeal CPs for which detailed X-ray crystal structures are known. It is demonstrated that CXMS (previously performed only for non-halophilic proteins of relatively neutral pI) coupled with atomic structural data (determined experimentally for non-halophilic protein homologs) provides useful distance constraints for extremely acidic halophilic protein complexes (proteasomal CP from the archaeon *H. volcanii*). Several observed cross-links were used to validate the predicted 3D-structures of the *H. volcanii* proteasomal α- and β -type subunits, and intermolecular distance constraints were used to assemble these proteins (α1, β) into the quaternary structure of the CP complexes. Distance constraints obtained by this CXMS study facilitated selection of an accurate model from several possible PDB proteasomal models and assisted in the determination of the arrangement of the α1- and β- homoheptameric rings in the complex protein structures.

Acknowledgements

This work was funded in part by: NIH R01 GM057498 and DOE DE-FG02-05ER15650 to JMF; 1S10RR023044-01 and 1U54AI57141-01 to DRG; Fulbright Association and MNRS-III 43004 to IMK. Authors thank Dr. Priska von Haller (Proteomics Resource, University of Washington) for her assistance with mass spectrometry analysis. Special thanks to Yi-Hsuan Tsai (University of North Carolina) for assistance with bioinformatics and Branko Drakulic (University of Belgrade) for homology modeling.

References

1. Singh, P., Panchaud, A., Goodlett, D.R. (2010). Chemical cross-linking and mass spectrometry as a low-resolution protein structure determination technique. *Anal. Chem*. **82**: 2636-42.
2. Lee, J. Y. (2008). Mass spectrometric analysis of cross-linking sites for the structure of proteins and protein complexes. *Mol. Biosyst*. **4**: 816-823.
3. Singh, P., Shaffer, S.A., Scherl, A., Holman C. *et al.*, (2008). Characterization of protein cross-links via mass spectrometry and an open-modification search strategy. *Anal. Chem.* **80**: 8799-8806.
4. Panchaud, A., Singh, P., Shaffer, S.A., Goodlett, D.R. (2010). xComb: a cross-linked peptide database approach to protein-protein interaction analysis. *J. Proteome Res*. **9**: 2508-2515.
5. Sinz A. (2006). Chemical cross-linking and mass spectrometry to map three-dimensional protein structures and protein-protein interactions. *Mass Spectrom. Rev*. **25**: 663-82.
6. Müller S, Bich C, Touboul D, Zenobi R. (2009). Chemical cross-linking with NHS esters: a systematic study on amino acid reactivities. *J. Mass Spectrom.* **44**: 694-706.
7. Mouradov, D., King, G., Ross, I. L., Forwood, J.K., *et al.*, (2008). Protein structure determination using a combination of cross-linking, mass spectrometry, and molecular modeling. *Methods Mol. Biol.* **426**:459-74.
8. Back, J.W., De Jong, L., Muijsers, A.O., De Koster, C.G. (2003). Chemical cross-linking and mass spectrometry for protein structural modeling. *J. Mol. Biol.* **331**:303-13.
9. Leitner, A., Walzthoeni, T., Kahraman, A., Herzog, F. *et al.*, (2010) Probing native protein structures by chemical cross-linking, mass spectrometry, and bioinformatics. *Mol. Cell Proteomics* **9**: 1634-49.
10. Schulz, D. M., Kalkhof, S., Schmidt, A., Ihling, C., *et al.*, (2007). Annexin A2/P11 interaction: new insights into annexin A2 tetramer structure by chemical cross-linking, high-resolution mass spectrometry, and computational modeling. *Proteins* **69:** 254–269.
11. Balasu, M. C., Spiridon, L. N., Miron, S., Craescu, C. T., *et al.*, (2009). Interface analysis of the complex between ERK2 and PTP-SL. *PLoS One,* **4:** e5432.
12. Heymann, M., Paramelle, D., Subra, G., Forest, E. *et al.*, (2008). MSX-3D: a tool to validate 3D protein models using mass spectrometry. *Bioinformatics* **24**: 2782-2783.
13. Zhou, G., Kowalczyk, D., Humbard, M.A., Rohatgi, S., *et al.,* (2008). Proteasomal components required for cell growth and stress responses in the haloarchaeon *Haloferax volcanii*. *J. Bacteriol*. **190**: 8096-8105.
14. Wilson, H.L., Aldrich, H.C., Maupin-Furlow, J.A. (1999). Halophilic 20S proteasomes of the archaeon *Haloferax volcanii*: purification, characterization, and gene sequence analysis. *J. Bacteriol.* **181**: 5814-5824.
15. Kaczowka, S.J., Maupin-Furlow, J.A. (2003). Subunit topology of two 20S proteasomes from *Haloferax volcanii*. *J. Bacteriol.* **185**: 165-174.
16. Lam, Y.A., Lawson, T., Velayutham, M., Zweier, *et al.*, (2006). A proteasomal ATPase subunit recognizes the polyubiquitin degradation signal. *Nature* **416**: 763-767.
17. Kao, A., Chiu, C., Vellucci, D., Yang, Y. *et al.*, (2011). Development of a novel cross-linking strategy for fast and accurate identification of cross-linked peptides of protein complexes. *Mol. Cell. Proteomics* **10**: 1-17.
18. Karadzic, I., Maupin-Furlow, J.A. (2005). Improvement of two-dimensional gel electrophoresis proteome maps of the haloarchaeon *Haloferax volcanii*. *Proteomics* **5**: 354-359.

19. Schilling, B., Row, R.H., Gibson, B.W., Guo, X. *et al.*, (2003). MS2Assign, automated assignment and nomenclature of tandem mass spectra of chemically crosslinked peptides. *J. Am. Soc. Mass. Spectrom.* **14**: 834-50.
20. Holm, L., Park, J. (2000). DaliLite workbench for protein structure comparison. *Bioinformatics* **16**: 566-567.
21. Pedretti, A., Villa, L., Vistoli, G. (2002). VEGA: a versatile program to convert, handle and visualize molecular structure on Windows-based PCs. *J. Mol. Graph. Model*. **21**: 47-49.
22. Leitner, A., Walzthoeni, T., Kahraman, A., Herzog, F. *et al.*, (2010). Probing native protein structures by chemical cross-linking, mass spectrometry,and bioinformatics. *Mol. Cell Proteomics* **9**: 1634-49.
23. Groll, M., Brandstetter, H., Bartunik, H., Bourenkow, G. *et al.*, (2003). Investigations on the maturation and regulation of archaebacterial proteasomes. *J. Mol. Biol.* **327**: 75-83.
24. Karadzic, I., Maupin-Furlow, J., Humbard, M., Prunetti, L., Singh, P., Goodlett, D. (2012) Chemical cross-linking, mass spectrometry, and *in silico* modeling of proteasomal 20S core particles of the haloarchaeon *Haloferax volcanii. Proteomics* 12: 1–9.
25. Humbard, M., Miranda, H., Lim, J-M., Krause, D., *et al.*, (2010). Ubiquitin-like small archaeal modifier proteins (SAMPs) in *Haloferax volcanii. Nature* 463: 54–60.
26. Zhang, F., Hu, M., Tian, G., Zhang P., *et al.,* (2009). Structural insights into the regulatory particle of the proteasome from *Methanocaldococcus jannaschii*. *Mol Cell.* 34:473-84.
27. Löwe**,** J**.**, Stock**,** D**.**, Jap**,** B**.**, Zwickl**,** P**.**, *et al.,* (1995). Crystal structure of the 20S proteasome from the archaeon *T. acidophilum* at 3.4 Å resolution. *Science* 268:533-9.

Application of structural biochemistry to the study of ion channel activation: structural basis of TRPP2 channel gating

Edward T. Petri[a*], Andjelka S. Celic[a], Michael Hodsdon[b], Titus J. Boggon[b] and Barbara E. Ehrlich[b]

[*] [a]*Department of Biology and Ecology, University of Novi Sad, Novi Sad, Serbia;*
[b]*Yale University School of Medicine, New Haven, CT, USA*
e-mail: Edward.Petri@dbe.uns.ac.rs

Transient Receptor Potential (TRP) channels are conserved from yeast to humans, and serve as cellular sensors in a wide-range of physiological processes. Little is known about how TRP channels transduce myriad stimuli into channel openings, and the structural changes governing TRP channel gating remain unknown. TRPP2 is a calcium (Ca^{2+}) permeable TRP channel whose activity rises and falls with cytoplasmic Ca^{2+} levels. Inherited mutations in polycystin-1 (PC1) and TRPP2 lead to improper function of the PC1-TRPP2 channel complex and polycystic kidney disease (PKD). In patients with PKD, PC1 and TRPP2 are frequently mutated or truncated in their C-terminal cytoplasmic tails, which mediate the PC1-TRPP2 interaction: two thirds of pathogenic mutations in PC1 and >90% of pathogenic mutations in TRPP2 result in truncations predicted to ablate these cytoplasmic regions and abrogate interaction between the proteins. We applied biochemical and biophysical methods to determine the structural basis of PKD associated mutations and present the first picture of TRPP2 channel gating. Here we describe a model where TRPP2 ion channels are gated by discrete conformational and oligomerization state changes in the C-terminal cytoplasmic tail in response to changes in cytoplasmic Ca^{2+} levels. We speculate that TRP channels in general are activated by stimuli-induced conformational changes in their N- or C-terminal extensions which are propagated to the ion channel pore, modulating channel activity.

Introduction

Autosomal Dominant Polycystic Kidney Disease (ADPKD) is a common systemic disorder that affects between 1 in 400 and 1 in 1000 individuals worldwide; and is characterized by renal and hepatic cysts, intracranial or aortic aneurysms, and mitral valve prolapse[1, 2]. Most cases of ADPKD (>95%) are caused by genetic mutations in either the *Pkd1* or the *Pkd2* gene, which encode polycystin-1 (PC1) and TRPP2, respectively. TRPP2 (a.k.a. polycystin-2 or TRPP2) is a six-transmembrane (6TM) Ca^{2+}-permeable TRP channel with cyto-

plasmic C-and N-termini; while PC1 is a large 11TM membrane protein whose function is less clear[2]. PC1 and TRPP2 interact directly and co-localize to primary cilia where they are hypothesized to be necessary for a mechanosensory or chemosensory response that triggers a rise in intracellular Ca^{2+}. In patients with ADPKD, inheritable mutations in polycystin-1 (PC1) and/or TRPP2 are believed to impair the function of the PC1-TRPP2 channel complex, leading to polycystic kidney disease. Many aspects of TRPP2 function are mediated by its' cytoplasmic C-terminal tail, including co-assembly with PC1 through the PC1 C-terminal cytoplasmic tail[3]. In ADPKD, PC1 and TRPP2 are frequently mutated or truncated in their C-terminal cytoplasmic tails: two thirds of pathogenic mutations in PC1 and >90% of pathogenic mutations in TRPP2 result in truncations which would disrupt functional domains within these cytoplasmic regions, abrogating the interaction between PC1 and TRPP2[3].

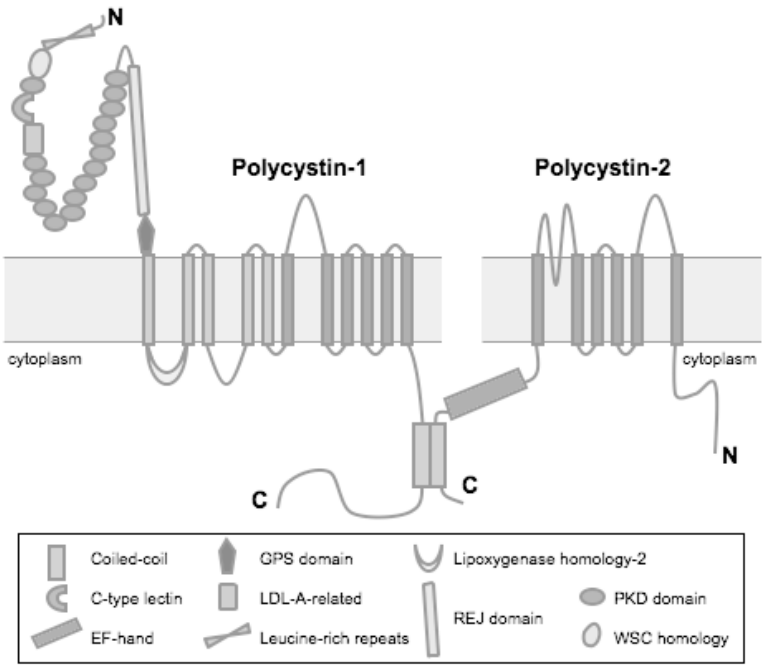


Figure 1. Schematic of the polycystin-1 polycystin-2 (TRPP2) complex

After their initial discovery as environmental sensors of chemical and physical stimuli, TRP channels have since been found to perform a wide range of physiological functions in all species from yeast to humans[4]. While all TRP channels are thought to be 6TM tetrameric channels, sequence conservation is somewhat limited beyond residues forming the predicted channel pore. No crystal structures have been solved of any TRP channels, and very little is known about how TRP channels are activated to open the channel pore for ion flux (*i.e.*

gating). However, a common theme among TRP channels is the presence of cytoplasmic extensions with various functional domains, such as ankyrin repeats, coiled coil motifs and EF-hand domains[5]. These widely variable cytoplasmic tails could serve as signaling sensors or switches, enabling TRP channels to be activated or modulated by specific stimuli, such as protein-protein interactions, changes in redox state[6-10], heat, or ligand binding. For example, both calcium[11] and reactive oxygen species (ROS) have been shown to regulate TRPP2 channels[12]. Such signals could then be transduced into conformational changes that open the channel pore. However, how these sensor domains communicate with the ion channel pore remains unknown.

Development of a Structural Model of the Ca^{2+} sensing region of TRPP2[13]

The original model of the C-terminal cytoplasmic tail of TRPP2 consisted of an EF-hand motif overlapping with a short coiled coil[14]. However, residues in the predicted coiled coil interface are hydrophilic or charged, and are unfavorable for coiled coil oligomerization. Moreover, ADPKD-associated truncation mutations have been identified outside of this region in TRPP2, suggesting that residues important for channel function are located in the truncated regions. In fact, TRPP2 binds directly to several proteins, including the inositol 1,4,5 trisphosphate receptor, TRPC1, tropomyosin-1, Id2, troponin-1, KIF3A and PC1(*2*): these interactions are dependent on residues C-terminal to the originally accepted domain model of the TRPP2 C-terminal tail. In order to address these discrepancies and begin to determine the molecular basis of TRPP2 channel gating and ADPKD pathogenesis and progression, we used a battery of biochemical and biophysical methods to construct a structural model of the C-terminal tail of TRPP2. We show that, like other TRP channels, the C-terminal cytoplasmic tail of TRPP2 contains several functional domains: an EF-hand domain connected by a flexible acidic linker to a previously unknown coiled coil domain[13]. Based on structural analysis and modeling of these domains, we hypothesized that these domains play important functional roles in Ca^{2+}-dependent channel activation and oligomerization in TRPP2.

We initially constructed a *de novo* molecular model of the C-terminal tail of TRPP2 (TRPP2-C) using the ROBETTA protein structure prediction suite, and validated characteristics of this model using biophysical and biochemical methods, including: limited proteolysis, MALDI mass spectroscopy, N-terminal sequencing, size-exclusion chromatography, analytical ultracentrifugation and circular dichroism spectroscopy. Based on these results, we show that the C-terminal tail of TPRP2 (TRPP2-C) contains two domains, a single EF-hand motif (TRPP2-EF) connected by a proteolyticaly sensitive linker to a coiled coil domain (TRPP2-CC). Our model suggests that the coiled coil domain is involved in TRPP2 channel oligomerization and that EF-hand could act as a Ca^{2+}-sensitive switch during Ca^{2+}-induced channel activation and inhibition. Importantly, PKD-associated truncation mutations are located in regions that would disrupt these functions, providing structural insight into how mutations in TRPP2 may lead to polycystic kidney disease.

Molecular modeling of the C-terminal tail of TRPP2 predicts two domains connected by a linker

De Novo structural models of TRPP2-C (I704-V968) and TRPP2-EF (K719-M800) were obtained from the ROBETTA protein structure prediction suite. The TRPP2-C model predicts an α-helical, two domain elongated structure connected by a linker containing a known TRPP2 phosphorylation site (S812)[15]. Domain-1 (~I704-S794) contains a canonical single EF-hand motif and a well-defined Ca^{2+}-binding site (TRPP2-EF), whereas domain-2 (~G828-V968) contains a striking ~40 residue long central α-helix (Y836-K876) characteristic of a coiled coil (TRPP2-CC). We therefore analyzed the sequence of TRPP2-C for coiled coil propensity using a stringent coiled coil prediction algorithm (MARCOIL). MARCOIL predicts that residues F839-D919 have a high probability to form a coiled coil, and heptad repeat residues ('a-d') were mapped and found to correspond to a hydrophobic stripe on the long α-helix in TRPP2-CC. Interestingly for TRPP2 function, residues within TRPP2-CC are necessary for binding to TRPP2 interacting proteins such as TRPC1, PC1 and KIF3A, as well as possibly channel oligomerization. Furthermore, TRPP2-CC is the site of pathogenic PKD-associated truncation mutations (such as R872X), which should be unable to interact with the C-terminus of PC1, leading to a plausible hypothesis for PKD pathogenesis.

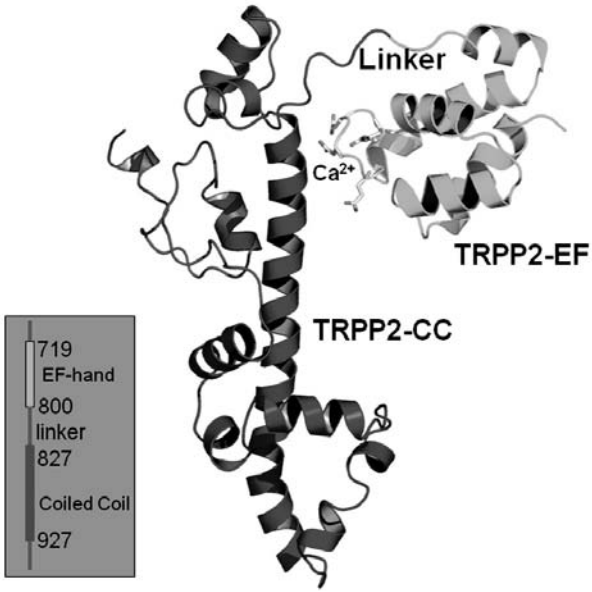


Figure 2.
Theoretical model of TRPP2-C

Limited proteolysis validates the structural model of the C-terminal tail of TRPP2

To test the validity of our structural model, we conducted limited proteolysis experiments coupled with MALDI-mass spectroscopy and N-terminal sequencing. TRPP2-C (I704-V968) was treated trypsin or proteinase K to identify proteolytically resistant fragments. Proteolytically resistant bands analyzed by N-terminal sequencing/MALDI-MS correspond to peptides beginning with 'ALVKL' (A711-L715) and 'NTVDD' (N720-D724) from trypsin digestion and 'SFPRS' (S804-S808) and 'SSRRR' (S823-R827) from proteinase K digestion, and identified two proteolytically resistant domains, A711-P797 and G828-H927. In addition, both proteinase K cleavage sites occur within the predicted linker region of our model, suggesting a flexible interdomain linker between residues R803 and H822. These domains identified by limited proteolysis overlap well with the positions of the predicted domains, validating our two domain model and the position of the linker region. Using these results we generated recombinant TRPP2-EF (N720-P797) and TRPP2-CC (G828-H927) for further biochemical and biophysical analysis.

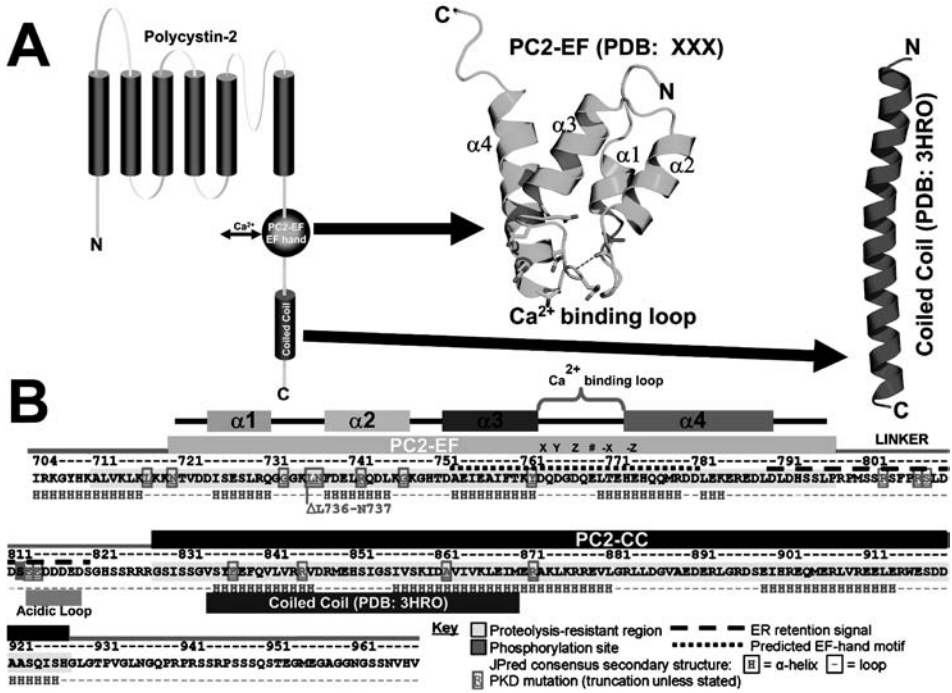


Figure 3.
Schematic of TRPP2 showing TRPP2-EF (PC2-EF) and TRPP2-CC (PC2-CC)

Circular dichroism shows that TRPP2-C undergoes Ca^{2+}-induced conformational changes

Our structural model of TRPP2-C predicts an all α-helical protein. To test this, we compared the secondary structure content of the TRPP2-C model with that obtained by circular dichroism (CD) spectroscopy. CD spectra were recorded and observed secondary structure content was compared with percentage α-helicity from our TRPP2-C model for TRPP2-C, TRPP2-EF and TRPP2-CC. In addition, because the model of TRPP2-C suggests a Ca^{2+}-binding site, we also investigated the Ca^{2+} sensitivity of the conformation of TRPP2-C. TRPP2-EF shows an increase from ~27% to ~45% α-helicity after Ca^{2+} addition, and TRPP2-C increases from ~34% to ~50% α-helicity with Ca^{2+}. These values for Ca^{2+}-bound TRPP2-C (~50%) and TRPP2-EF (~45%) correspond well with our structural models of TRPP2-C (62%) and TRPP2-EF (51%). Secondary structure content calculated from CD spectra for TRPP2-CC (~70% α-helix) is also in agreement with our structural model of the coiled coil domain (74%). CD thus validates the fold predicted by our model and suggests that Ca^{2+} binding induces a conformational change within the EF-hand domain.

TRPP2-C contains a Ca^{2+}-binding EF-hand domain by isothermal titration calorimetry

Our structural model and CD analysis suggested that the TRPP2 EF-hand domain could bind Ca^{2+}. To verify and quantify this Ca^{2+} binding, we conducted isothermal titration calorimetry (ITC). Fitting ITC isotherms with a 1-site binding model indicates that TRPP2-C binds Ca^{2+} with a K_d of ~12 μM, whereas TRPP2-EF binds Ca^{2+} with lower affinity (KB_{dB}~214 μM) and 1:1 stoichiometry. These different affinities for Ca^{2+} imply that regions outside of TRPP2-EF are involved in stabilization or regulation of the Ca^{2+} binding site. As a control, TRPP2-EF with mutations in the Ca^{2+} binding loop (T771A/E774A) did not bind Ca^{2+}.

Analytical ultracentrifugation suggests that TRPP2 channel oligomerization is mediated by the coiled coil domain

To examine the oligomerization state and molecular conformation of TRPP2-C, TRPP2-EF and TRPP2-CC, we performed high speed sedimentation velocity ultracentrifugation experiments. AUC resolves heterogeneity in solution according to shape and molecular weight. Velocity sedimentation results suggest that TRPP2-CC is mostly present as an oligomer with an elongated shape, and that TRPP2-EF is monomeric with a globular shape. These results support a model of TRPP2-C as an oligomeric elongated macromolecule whose oligomerization is mediated by the C-terminal coiled coil domain, TRPP2-CC.

NMR structure determination of the Ca^{2+} sensing region of TRPP2 channels[16]

Based on the above results, we proposed that TRPP2-EF is a Ca^{2+}-sensor that regulates TRPP2 activity, and that the coiled coil mediates channel oligomerization. X-ray crystal

structure determination of the TRPP2 coiled coil (833-895)[17] confirmed part of our model, but investigation was still required into the structural basis of TRPP2 regulation by Ca^{2+}.

Several Ca^{2+}-dependent channels contain EF-hand domains in their C-terminal tails which modulate channel activity. For example, an EF-hand mutation in $Na_V1.4$ channels disrupts fast channel inactivation, and bestrophin Cl^- and BK channels contain EF-hands which, when mutated, abolish Ca^{2+}-sensitivity. We propose that Ca^{2+}-binding to the C-terminal EF-hand in TRPP2 mediates the Ca^{2+}-dependence of TRPP2 channel activity. TRPP2 channels are activated at low levels of Ca^{2+}, reach a maximum with increasing concentrations and are inactivated at higher levels. Although phosphorylation of TRPP2 (at S812) raises the threshold [Ca^{2+}] required for TRPP2 activation, Ca^{2+}-dependence curves for non-phosphorylatable TRPP2 mutants (S812A) remain bell-shaped. Thus, another mechanism must be responsible for conferring Ca^{2+}-dependence to TRPP2. Interestingly, the [Ca^{2+}] required to inhibit S812A TRPP2 channels is ~equal to the K_d of Ca^{2+}-binding to TRPP2-C. Moreover, ADPKD mutations N720X, ΔL736-N737, R742X, and Y762X have been mapped to TRPP2-EF (see http://pkdb.mayo.edu), while in cultured cells, cytoplasmic Ca^{2+} levels are regulated by TRPP2; an effect abrogated in R742X, suggesting a crucial role for Ca^{2+}-binding by the EF-hand. Thus, to describe the role of the EF-hand in Ca^{2+}-dependent regulation of TRPP2 channels and the structural basis of TRPP2-EF Ca^{2+}-binding, we determined the NMR structure and dynamics of Ca^{2+}-bound TRPP2-EF.[16]

NMR structure determination confirmed that TRPP2-EF does contain a single Ca^{2+}-binding site in an EF-hand motif comprising the α3-helix, Ca^{2+}-binding loop, and α4-helix. A helix-loop-helix (HLH) consisting of helices α1 and α2 is paired with this EF-hand as predicted by our structural model of TRPP2-EF. However, in contrast with canonical EF hands, which contain two Ca^{2+}-binding sites, the α1-α2 loop (G732-N737) 'GGGKLN' does not contain the residues necessary for Ca^{2+}-coordination. Interestingly, ancestral invertebrate TRPP2 channels, such as in sea urchins, appear to contain a canonical dimeric EF-hand with two Ca^{2+}-binding sites; the first corresponding to α1-α2 in human TRPP2-EF and the second, α3-α4. Sea urchin TRPP2, which is involved in fertilization, has a different localization and function than human TRPP2, and the evolutionary loss of the first Ca^{2+}-binding site may have adapted human TRPP2 for its' new functions. Structurally, this adaptation would reduce Ca^{2+}-affinity, and increase conformational flexibility, enabling Ca^{2+}-sensitive regulation of human TRPP2. In addition, an ADPKD pathogenic mutation ΔL736-N737 was identified which lies within the α1-α2 loop and likely destabilizes the α3-α4 Ca^{2+}-binding loop. Whereas ADPKD truncation mutations N720X, R742X, and Y762X eliminate the EF-hand and coiled coil, ΔL736-N737 should affect only the EF-hand, implying its functional importance. Based on this structure, we propose a model where Ca^{2+}-binding to one EF-hand leads to conformational changes which stabilize the EF-hand from neighboring C-terminal tails in tetrameric TRPP2 channels, leading to cooperative Ca^{2+}-binding and activation of TRPP2 channel activity, followed by inhibition upon saturation of available Ca^{2+} binding sites.

The structural basis of TRPP2 channel gating[18]

The NMR structure of Ca^{2+}-bound TRPP2 EF-hand revealed a single Ca^{2+}-binding site[16]; while Isothermal Titration Calorimetry showed that this domain binds Ca^{2+} non-cooperatively[13]. In the context of the entire C-terminal cytoplasmic tail, however, the affinity for Ca^{2+} is increased, and the mode of Ca^{2+}-binding is cooperative, suggesting synergistic interactions between the EF-hand and coiled coil domains during TRPP2 channel gating.

TRPP2 ion channel activity is bell-shaped with respect to Ca^{2+}, with activation by low levels of cytoplasmic Ca^{2+} and inhibition at higher Ca^{2+} concentrations. Furthermore, phosphorylation of the acidic linker at S812 in the TRPP2 C-terminal tail modulates the threshold concentration of Ca^{2+} required for channel activation, and the Ca^{2+} dependence of channel activity requires an intact C-terminal cytoplasmic tail[15]; furthermore, TRPP2 channel activity can be altered by interactions with other proteins, such as PC1[3]. Together, these data suggest that TRPP2 channel gating is mediated by its' C-terminal cytoplasmic tail. However, the molecular basis for these regulatory mechanisms remains unknown.

Below, we show by Small-Angle X-ray Scattering (SAXS), NMR and Analytical Ultracentrifugation (AUC) that Ca^{2+}-binding by the TRPP2 EF-hand domain induces discrete conformational and oligomerization state transitions in the entire C-terminal cytoplasmic region. Moreover, we demonstrate that mutant TRPP2 channels unable to bind Ca^{2+} are inactive in single-channel planar lipid bilayers, and inhibit Ca^{2+} release from endoplasmic reticulum stores.[18]

The TRPP2 EF-hand domain undergoes global conformational changes upon binding Ca^{2+}

In order to serve as a Ca^{2+} sensitive channel regulator, the TRPP2 EF-hand domain must respond structurally to physiological changes in cytoplasmic Ca^{2+}. To test if TRPP2-EF undergoes a global conformational change upon Ca^{2+} binding, we conducted SAXS on the TRPP2 EF-hand domain. SAXS provides solution state analysis of macromolecular envelope changes (*i.e.* global conformational changes), for example in response to ligand binding. SAXS experiments were conducted on TRPP2-EF in both a Ca^{2+}-free state and in the presence of excess Ca^{2+}. A dramatic reduction in the radius of gyration, R_g (from ~30 Å to ~22 Å) was observed between Ca^{2+}-bound and Ca^{2+}-free TRPP2-EF. In addition, the maximum interatomic distances, D_{max}, for TRPP2-EF reduced from 84 Å to 61 Å on addition of Ca^{2+}. Moreover, a*b initio* SAXS envelope reconstructions correspond very well to the NMR structure of Ca^{2+}-bound TRPP2-EF (PDB ID: 2K6Q), while SAXS envelope reconstructions for the Ca^{2+}-free TRPP2-EF suggest a mostly unfolded protein.

Ca^{2+} binding induces a folding state transition in the TRPP2 EF-hand

To determine if Ca^{2+} induced conformational changes correspond to a discrete structural transition consistent with ion channel gating regulation, residue specific structural changes in TRPP2-EF on titration with Ca^{2+} were investigated by NMR. Resonance-specific chemical shifts were assigned for apo-TRPP2-EF using conventional NMR methods and compared with chemical shift assignments for Ca^{2+}-bound TRPP2-EF. Then, using the ^{13}C chemical shift index (CSI) and C_α chemical shift assignments, CSI changes between apo and Ca^{2+}-bound TRPP2-EF were mapped onto the NMR structure of Ca^{2+}-bound TRPP2-EF. Significant conformational changes occur in helices α1, α3 and α4 of TRPP2-EF on binding Ca^{2+}. These changes correspond to residues in the C-terminus of α3 (I758-Y762), the Ca^{2+}-binding loop between α3 and α4 (D763-T771) and residues at the N-terminus of α4 (E772-K784). Based on these results, the TRPP2 EF-hand domain appears to undergo a folding-unfolding transition on binding Ca^{2+}, consistent with its proposed role as a Ca^{2+} sensor in TRPP2 channel gating.

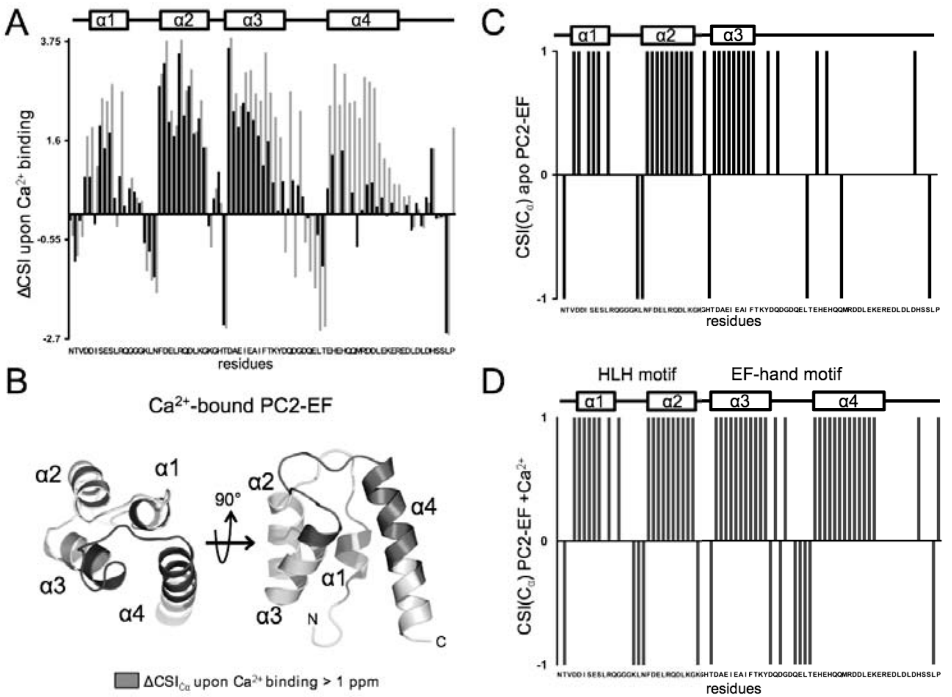


Figure 4. Ca^{2+} induced conformation changes in TRPP-EF (PC2-EF)

The C-terminal tail of TRPP2 transitions from an extended oligomer to a compact dimer upon binding Ca^{2+}

To serve as a Ca^{2+} sensitive channel gating module, the entire C-terminal cytoplasmic tail of TRPP2 should undergo discrete structural changes in response to changes in physiological cytoplasmic Ca^{2+} levels. To investigate changes in the molecular shape and oligomerization state of TRPP2-C upon Ca^{2+} binding, we again conducted high-speed velocity sedimentation experiments on TRPP2-C by AUC. We performed three experiments, at 0 mM, 100 µM and 5 mM Ca^{2+}, approximately corresponding to 0:1, 1:1 and 50:1 ratios of Ca^{2+} to TRPP2-C (94 µM). We then investigated the frictional ratios of TRPP2-C species present in the 1:1 and 50:1 Ca^{2+} conditions. Frictional ratios depend on the molecular shape of a sample, where an f/f_o of ~2 indicates an elongated particle, and f/f_o of ~1 indicates a compact, spherical shape. For the 1:1 Ca^{2+} condition, 2DSA analysis indicates that TRPP2-C (MW 32.5 kDa) was in three distinct states: 1) Extended tetramer, with an f/f_o ~2.1 and a molecular weight ~120-130 kDa; 2) Extended trimer, with an f/f_o of ~1.9 and molecular weight ~100-110 kDa; and 3) Compact dimer, with an f/f_o of ~1.3 and molecular weight ~65-70 kDa. However, remarkably, in the presence of excess Ca^{2+} (50:1 condition), a single compact dimer was observed (f/f_o of ~1.3 and molecular weight ~65-70 kDa). Thus, the C-terminal cytoplasmic tail undergoes discrete conformational and oligomerization state changes in response to changes in Ca^{2+} levels, consistent with its proposed role as a Ca^{2+} sensitive gating module.

Ca^{2+} induced conformational changes in the TRPP2 EF-hand are propagated throughout the C-terminal tail

To regulate channel gating, the TRPP2 EF-hand domain must be able to sense changes in cytoplasmic Ca^{2+} and transduce those changes to the entire TRPP2 channel. To determine if the conformational changes observed for the TRPP2 EF-hand alone are propagated throughout the entire C-terminal cytoplasmic tail of TRPP2, we conducted SAXS analysis on TRPP2-C in both the Ca^{2+}-free state, and in the presence of excess Ca^{2+}. Similar to our SAXS results for the isolated TRPP2 EF-hand, a significant change in R_g (from ~72 Å to ~62 Å) and D_{max} was observed between Ca^{2+}-bound and Ca^{2+}-free TRPP2-C. These significant reductions in Rg and D_{max} in the presence of excess Ca^{2+} correlate with a compression of the overall structure of TRPP2-C that could be mediated by Ca^{2+}-induced folding and inter-domain interactions, in agreement with our AUC results described above.

Overall, our AUC and SAXS data demonstrate that the global conformation of TRPP2-C transitions from a mixture of extended oligomers to a single compact dimer in the presence of increasing Ca^{2+} concentrations. These results are consistent with a role for the TRPP2 C-terminal cytoplasmic tail in regulating TRPP2 channel gating in response to changes in cytoplasmic Ca^{2+} levels.

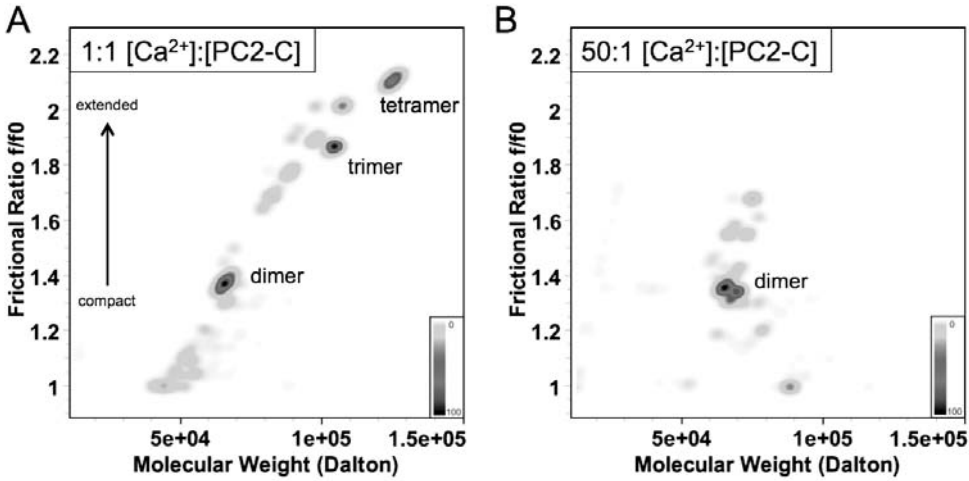


Figure 5. Discrete Ca^{2+}-induced conformation changes in TRPP-C (PC2-C)

Ca^{2+} binding by the TRPP2 EF-hand is required for single TRPP2 channel activity in planar lipid bilayers

We previously measured the single-channel properties of wildtype TRPP2 channels in response to increasing concentrations of Ca^{2+}. If Ca^{2+} induced conformational changes in the TRPP2 EF-hand domain are required for channel gating, elimination of the Ca^{2+} binding in TRPP2-EF should affect the single channel properties of TRPP2 channels. To test this, we created a TRPP2 construct (TRPP2-x-z) with a mutated EF-hand domain (T771A/E774A) that is unable to bind Ca^{2+}. Microsomes were then prepared from cells overexpressing either TRPP2-x-z or wildtype TRPP2. Single channel experiments were conducted using multiple TRPP2-x-z containing samples and were performed in parallel on wildtype TRPP2 channels. No channel activity was observed in microsomes prepared from cell overexpressing TRPP2-x-z when the cytoplasmic free Ca^{2+} concentration was 0.1 um, a concentration at which wildtype TRPP2 channels were active. The Ca^{2+} dependence of channel activity showed that TRPP2-x-z channels were not active at Ca^{2+} concentrations for which wildtype TRPP2 channels display peak activity (n=5 for each condition). Additional single channel bilayer experiments were performed on TRPP2-x-z channels using a range of voltages (0-30 mV) known to activate wild type channels and no activity was observed. Together, these results strongly suggest that Ca^{2+} binding to the TRPP2 EF-hand is required for TRPP2 channel activity.

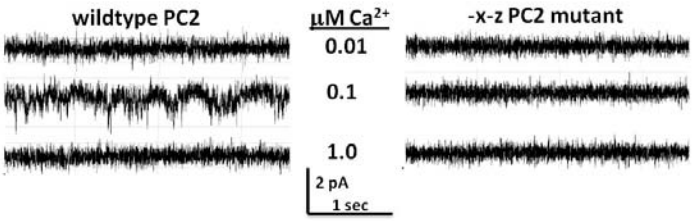


Figure 6. TRPP2 channel activity depends on Ca^{2+} binding

Expression of TRPP2-x-z channels inhibits Ca^{2+} release from intracellular stores

We have previously shown that TRPP2 functions as a Ca^{2+} release channel in the ER membrane. TRPP2 channels are believed to be homo-tetramers, with the C-terminal cytoplasmic tail forming oligomeric inter-tail interactions. To further investigate the role of Ca^{2+} binding by the TRPP2 C-terminal tail in TRPP2 channel function, we conducted live cell imaging of cytoplasmic Ca^{2+} levels in cells expressing either TRPP2-x-z or wildtype TRPP2. Although overexpression of wildtype TRPP2 was associated with increased cytoplasmic Ca^{2+} levels upon stimulation of ER Ca^{2+} release, expression of TRPP2-x-z resulted in a significant reduction in the amplitude and duration of Ca^{2+} release transients. Thus TRPP2-x-z does not appear to be functional, and does not contribute to ER Ca^{2+} release. In fact, TRPP2-x-z may behave as a dominant negative under these conditions, possibly by disrupting the C-terminal tail conformational and oligomerization state changes required for TRPP2 channel gating.

Conclusions

Addition of Ca^{2+} to the C-terminal cytoplasmic region of TRPP2 results in conformational transitions and oligomerization state changes, initiated by Ca^{2+} binding to the EF-hand domain, and Ca^{2+} binding by the EF-hand is necessary for TRPP2 channel activity. Changes in cytoplasmic Ca^{2+} levels induce discrete changes to the conformation and oligomerization state of the cytoplasmic region of TRPP2, which correlate with TRPP2 channel gating, suggesting a mechanism for TRPP2 channel regulation by protein binding to the C-terminal cytoplasmic region of TRPP2. Our model explains the pathogenicity of ADPKD associated truncation mutations, and our results support our hypothesis that TRPP2 channels are gated by discrete conformational and oligomerization state changes in the C-terminal cytoplasmic tail in response to changes in cytoplasmic Ca^{2+} levels. We propose that TRP channels in general are activated by similar, stimuli-induced conformational changes in their N- or C-terminal extensions which are propagated to the channel pore.

Acknowledgements

This work was funded by the Ministry of Education, Science and Technological Development of the Republic of Serbia, grant No. 173014 (E.T.P. and A.C. are also supported by grant No. 172021). This work was funded by NIH grants R01DK087844 and P50DK057328.

References

1. Kottgen, M. (2007) TRPP2 and autosomal dominant polycystic kidney disease, *Biochim Biophys Acta 1772*, 836-850.
2. Delmas, P. (2005) Polycystins: polymodal receptor/ion-channel cellular sensors, *Pflugers Arch 451*, 264-276.
3. Casuscelli, J., Schmidt, S., DeGray, B., Petri, E. T., Celic, A., Folta-Stogniew, E., Ehrlich, B. E., and Boggon, T. J. (2009) Analysis of the cytoplasmic interaction between polycystin-1 and polycystin-2, *Am J Physiol Renal Physiol 297*, F1310-1315.
4. Nilius, B., and Owsianik, G. (2011) The transient receptor potential family of ion channels, *Genome Biol 12*, 218.
5. Li, M., Yu, Y., and Yang, J. (2011) Structural biology of TRP channels, *Adv Exp Med Biol 704*, 1-23.
6. Ghelfi, E., Rhoden, C. R., Wellenius, G. A., Lawrence, J., and Gonzalez-Flecha, B. (2008) Cardiac oxidative stress and electrophysiological changes in rats exposed to concentrated ambient particles are mediated by TRP-dependent pulmonary reflexes, *Toxicol Sci 102*, 328-336.
7. Miller, B. A. (2006) The role of TRP channels in oxidative stress-induced cell death, *J Membr Biol 209*, 31-41.
8. Groschner, K., Rosker, C., and Lukas, M. (2004) Role of TRP channels in oxidative stress, *Novartis Found Symp 258*, 222-230; discussion 231-225, 263-226.
9. Agam, K., von Campenhausen, M., Levy, S., Ben-Ami, H. C., Cook, B., Kirschfeld, K., and Minke, B. (2000) Metabolic stress reversibly activates the Drosophila light-sensitive channels TRP and TRPL in vivo, *J Neurosci 20*, 5748-5755.
10. Balzer, M., Lintschinger, B., and Groschner, K. (1999) Evidence for a role of Trp proteins in the oxidative stress-induced membrane conductances of porcine aortic endothelial cells, *Cardiovasc Res 42*, 543-549.
11. Koulen, P., Cai, Y., Geng, L., Maeda, Y., Nishimura, S., Witzgall, R., Ehrlich, B. E., and Somlo, S. (2002) Polycystin-2 is an intracellular calcium release channel, *Nat Cell Biol 4*, 191-197.
12. Montalbetti, N., Cantero, M. R., Dalghi, M. G., and Cantiello, H. F. (2008) Reactive oxygen species inhibit polycystin-2 (TRPP2) cation channel activity in term human syncytiotrophoblast, *Placenta 29*, 510-518.
13. Celic, A., Petri, E. T., Demeler, B., Ehrlich, B. E., and Boggon, T. J. (2008) Domain mapping of the polycystin-2 C-terminal tail using de novo molecular modeling and biophysical analysis, *J Biol Chem 283*, 28305-28312.
14. Qian, F., Germino, F. J., Cai, Y., Zhang, X., Somlo, S., and Germino, G. G. (1997) PKD1 interacts with PKD2 through a probable coiled-coil domain, *Nat Genet 16*, 179-183.
15. Cai, Y., Anyatonwu, G., Okuhara, D., Lee, K. B., Yu, Z., Onoe, T., Mei, C. L., Qian, Q., Geng, L., Wiztgall, R., Ehrlich, B. E., and Somlo, S. (2004) Calcium dependence of polycystin-2 channel activity is modulated by phosphorylation at Ser812, *J Biol Chem 279*, 19987-19995.

16. Petri, E. T., Celic, A., Kennedy, S. D., Ehrlich, B. E., Boggon, T. J., and Hodsdon, M. E. (2010) Structure of the EF-hand domain of polycystin-2 suggests a mechanism for Ca2+-dependent regulation of polycystin-2 channel activity, *Proc Natl Acad Sci U S A 107*, 9176-9181.
17. Yu, Y., Ulbrich, M. H., Li, M. H., Buraei, Z., Chen, X. Z., Ong, A. C., Tong, L., Isacoff, E. Y., and Yang, J. (2009) Structural and molecular basis of the assembly of the TRPP2/PKD1 complex, *Proc Natl Acad Sci U S A 106*, 11558-11563.
18. Celic, A. S., Petri, E. T., Benbow, J., Hodsdon, M. E., Ehrlich, B. E., and Boggon, T. J. (2012) Calcium-induced conformational changes in C-terminal tail of polycystin-2 are necessary for channel gating, *J Biol Chem 287*, 17232-17240.

Cold enzyme hydrolysis of starch

Nataša Božić*

* *Centre for Chemistry, Institute of Chemistry, Technology and Metallurgy, University of Belgrade, Belgrade, Serbia.*
e-mail: nbozic@chem.bg.ac.rs

With efforts to reduce global reliance on fossil fuels and lower the greenhouse gas emission, an increasing search for renewably sourced materials, which can be used as feedstock for biofuel production, is ongoing in the past few decades. At the present, ethanol is the most common alternate fuel and is already produced on a fair scale, representing a sustainable substitute for gasoline in passenger cars. Basically, in the United States ethanol is produced by fermenting starch crops that have been converted into simple sugars, and the major feedstock for this fuel is corn. In Brazil ethanol is produced through the fermentation of sugar cane molasses. Various countries have been increasing their ethanol production as well, such as India (using sugar cane), Thailand (cassava), France (sugar beet), China (corn) and Canada (wheat), among others.
Improved molecular disassembly and depolymerization of grain starch to glucose are key to reducing energy use in the bioconversion of glucose to chemicals, ingredients, and fuels. In fuel ethanol production, these biorefining steps use 10-20% of the energy content of the fuel ethanol. The need to minimize energy use and to raise the net yield of energy can be met by replacing high-temperature, liquid-phase, enzymatic digestion with low temperature, solid-phase, enzymatic digestion. Also called cold hydrolysis, the approach is a step toward a "green" method for the production of fuel ethanol.

Introduction

Starch is the most important carbon and energy source among plant carbohydrates, and it is the second following cellulose in total biosynthesis[1]. Starch represents an inexpensive source for production of glucose, fructose and maltose syrups[2] and for obtaining the products of their fermentation; including food ingredients, biofuels, organic acids and other valuable compounds for industrial applications. Besides agricultural crops, starch is a significant component of domestic and commercial wastes and these could become useful resources to be converted into ethanol.

The disassembly and depolymerization of grain starch to glucose are the result of the hydrolysis of α-1,4- and α-1,6-linkages between glucose monomers. Acid hydrolysis was used for this from its discovery in 1813 at least until the 1970s. However, the dilute acid

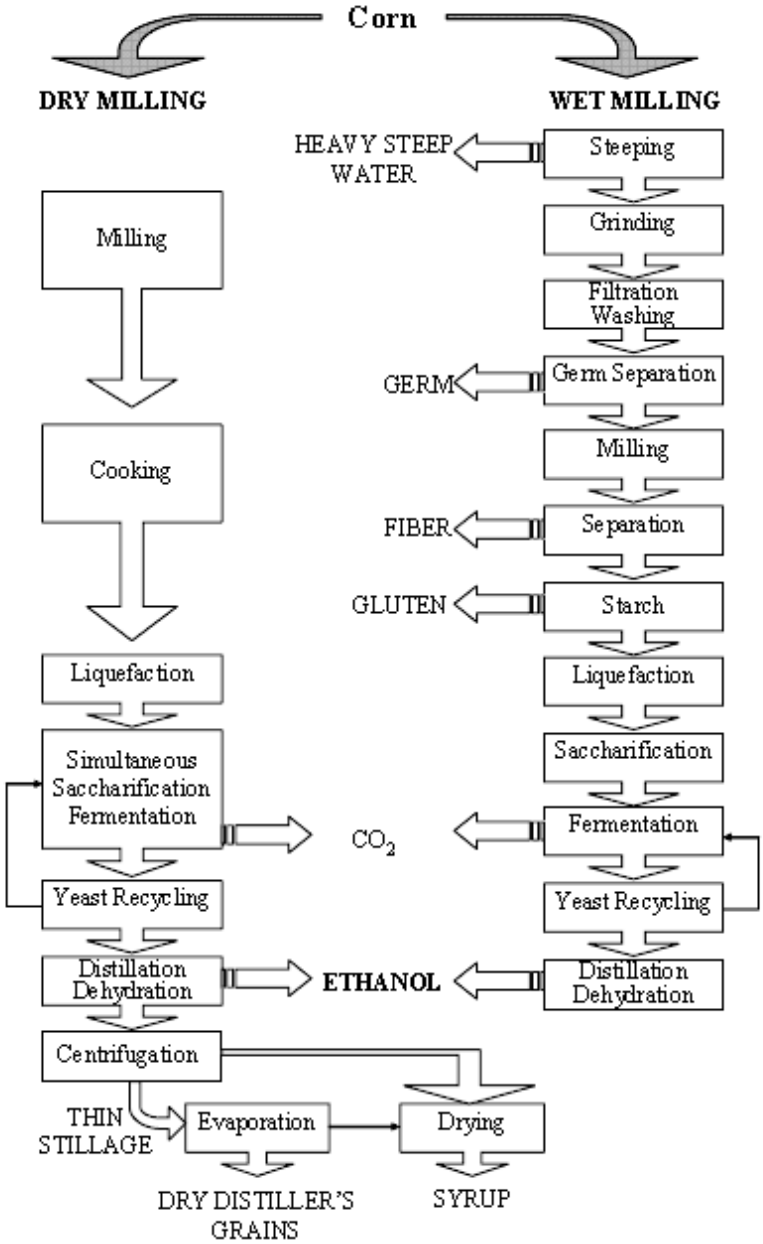


Figure 1.
Representation of dry milling and wet milling processes for bioethanol production[12]

and 120-150°C temperatures used in this process corroded equipment, formed undesirable byproducts, limited yield, and was costly[3-8]. High-temperature, liquid-phase enzymatic hydrolysis is now used for starch hydrolysis. One basic enzymatic hydrolysis configuration is a three-step sequence. In the first step, a 30% (by weight) slurry is cooked in the presence of α-amylase to 90-165 °C, cooled if necessary, held at 90 °C for 1-3 h, and then cooled further to 60 °C with the addition of glucoamylase. An energy-conserving alternative is to lower the starch-to glucose processing temperature below the onset of gelatinization which is for example, 54 °C for wheat, 60 °C for potato, or 65 °C for maize[6]. Regarding energy costs, effective utilization of natural resources, minimization of the formation of pollutants and viscosity (handling) problems, use of raw starch digesting enzymes that can perform direct hydrolysis of raw starch below gelatinization temperature is desirable[9]. The removal of the cooking stage also has the potential to increase the value of the co-products since valuable proteins would undergo less thermal stress[10].

Traditional production processes of ethanol from starch crops

Two different processes can be used to produce ethanol from starch crops: dry grind and wet milling, depicted in Figure 1. In dry grind, the feed material is ground mechanically and cooked in water to gelatinize the starch. Enzymes are then added to break down the starch to form glucose, which yeasts ferment to ethanol. In that case, a fixed amount of ethanol is produced, along with other feed products and carbon dioxide, and has almost no process flexibility. In wet milling, the insoluble protein, oil, fiber, and some solids are removed initially, remaining only the starch slurry fed to the ethanol production step. This process has the capability to produce various end products and considerable higher process flexibility, compared to the dry milling[11]. However, about 65% of the ethanol in the US is produced from dry grind corn processing plants[12], since initial investment in plant is 2 – 5 times cheaper.

Cold hydrolysis of starch

In addition to the most traditional processes for the production of ethanol from starchy materials, a nonconventional technology, named cold hydrolysis, has been investigated. Although the concept is not recent, since it was reported as a consequence of studies during the World War II[13,14], its application at large scale was demonstrated only recently[15,16]. The production of ethanol by cold hydrolysis of starch dispenses some of the steps of high energy demand in a plant, i.e., cooking and liquefaction. In this process, the raw (granular, non-cooked) starch is submitted to an initial hydrolysis step, in the presence of endoamylolytic and proteolytic preparations, so that it becomes more susceptible to saccharification. Unlike energy-demanding steps in traditional processes, the hydrolysis initiates at a temperature below that for the gelatinization of starch, for a few hours. The addition of proteases aims at to improve starch exposure by breaking down proteins associated to its polysaccharides[17,18]. The suspension, still rich in starch, goes to the fermenters, where more enzymes are added, mainly acid glucoamylases which are able to digest raw starch. The yeast is added to the vessel, so that the fermentation starts occurring simultaneously to the saccharification[19], Figure 2.

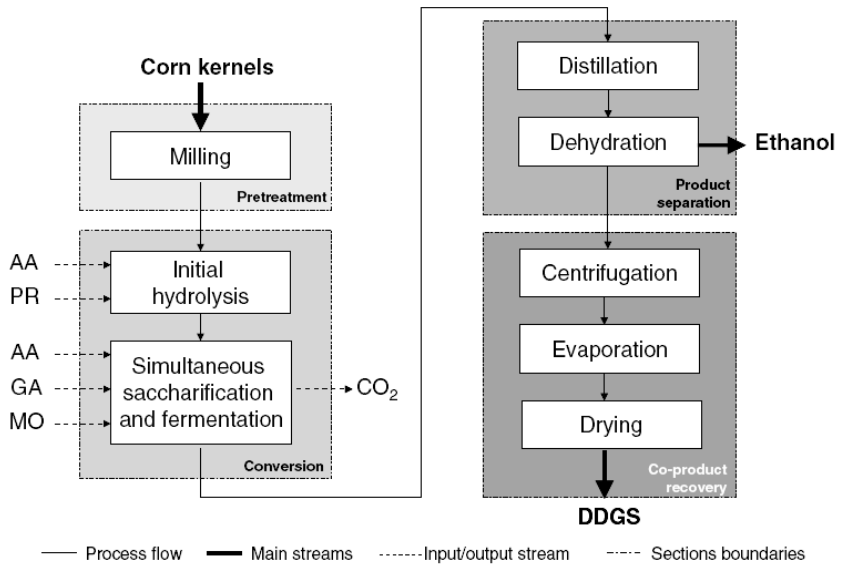


Figure 2. Simplified representation of cold hydrolysis process for production of ethanol from starch. AA α-amylase, PR protease, GA glucoamylase, MO microorganism, DDGS Distillers' Dried Grains with Solubles[20].

Besides the great energetic advantage of the cold hydrolysis process over the conventional technologies, the former also presents reduced water and chemicals consumption. The capital expenditure of a plant for conversion of raw starch is potentially lower, since the process is more integrated. The overall yield tends to be higher, due to the absence of Maillard reactions and reduced yeast inhibition. Since the sugars are gradually released, the cells tend to produce lower levels of coproducts, such as glycerol and higher alcohols and the osmotic stress is reduced[20]. Nevertheless, some drawbacks of the production of ethanol by cold hydrolysis includes: higher demand for enzymes (in both quantity and types of enzymes), since the hydrolysis of native starch presents some mass transfer limitations[21-23], which are present in a less extent or are nonexistent in the traditional processes; higher susceptibility for microbial contamination (by phytopathogens), which is avoided in the conventional technologies due to the high-temperature steps[24].

Starch structure

After its extraction from plants, starch occurs as a flour-like white powder insoluble in cold water. This powder consists of microscopic granules with diameters ranging from 2 to 100 μm, and with different size, shape, and chemical content depending on the botanic origin. Starch consists of mainly two glucosidic macromolecules: amylose and amylopectin. In

most common types of starch the weight percentages of amylose range between 72 and 82%, and the amylopectins range from 18 to 28%. However, some mutant types of starch have very high amylose content (up to 70% and more for amylomaize) and some very low amylose content (1% for waxy maize). Amylose is defined as a linear molecule of glucose units linked by (1-4) α-D-glycoside bonds, slightly branched by (1-6) α-linkages. Amylopectin is a highly branched polymer consisting of relatively short branches of α-D-(1-4) glycopyranose that are interlinked by α-D-(1-6)-glycosidic linkages approximately every 22 glucose units[25]. The multiplicity in branching lead Peat et al.[26] to describe the basic organization of the chains in terms of A, B and C chains. The single C chain per molecule, with a mean degree of polymerization (DP) above 60, carries other chains as branches and contains the terminal reducing end of the amylopectin macromolecule. The A chains are glycosidically linked to the rest of the molecule by their reducing group trough C6 of a glucose residue. The B chains are defined as bearing other chains as branches. They are linked to the rest of the molecule by their reducing group on one side and by a α-(1-6) linkage on the other, thus being the backbone of the grape-like macromolecule. From then, several models have been proposed, all referring to the cluster model.

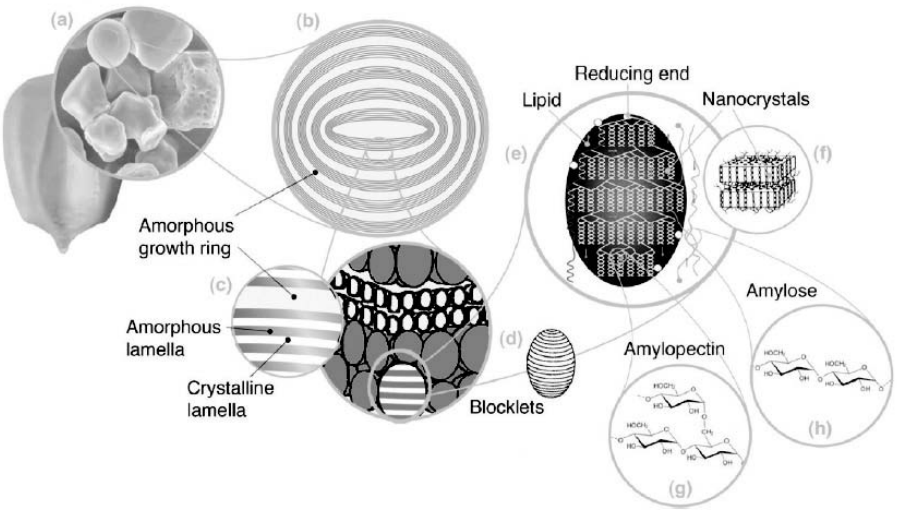


Figure 3. Starch multiscale structure: (a) starch granules from normal maize (30 μm), (b) amorphous and semicrystalline growth rings (120-500 nm), (c) amorphous and crystalline lamellae (9 nm), magnified details of the semicrystalline growth ring, (d) blocklets (20-50 nm) constituting a unit of the growth rings, (e) amylopectin double helixes forming the crystalline lamellae of the blocklets, (f) nanocrystals: other representation of the crystalline lamellae called starch nanocrystals when separated by acid hydrolysis, (g) amylopectin's molecular structure, and (h) amylose's molecular structure (0.1-1 nm)[27].

Minor components associated with starch granules are of three types: (i) cell-wall fragments, (ii) surface components, and (iii) internal components. The main constituents of surface components are proteins, enzymes, amino acids, and nucleic acids, whereas internal components are composed mainly of lipids. The proportion of these components depends on the botanical origin.

Starch structure has been under research for years, and because of its complexity, a universally accepted model is still lacking[27]. However, in this past decade a model seems predominant. It is a multiscale structure, shown in Figure 3, consisting of the (a) granule (2-100 μm) into which we find (b) growth rings (120-500 nm) composed of (d) blocklets (20-50 nm) made of (c) amorphous and crystalline lamellae (9 nm) containing (g) amylopectin and (h) amylose chains (0.1-1 nm). Starch granules consist of concentric alternating amorphous and semicrystalline growth rings.

α-Amylases

α-Amylases (E.C. 3.2.1.1.) are starch-degrading enzymes that catalyze the hydrolysis of internal α-1,4-*O*--glycosidic bonds in polysaccharides with the retention of α-anomeric configuration in the products. Most of the α-amylases are metalloenzymes, which require calcium ions (Ca^{2+}) for their activity, structural integrity and stability. They belong to family 13 (GH-13) of the glycoside hydrolase group of enzymes[28]. Amylases are one of the most important industrial enzymes that have a wide variety of applications ranging from conversion of starch to sugar syrups, to the production of cyclodextrins for the pharmaceutical industry. These enzymes account for about 30% of the world's enzyme production[29]. The α-amylase family can roughly be divided into two groups: the starch hydrolyzing enzymes and the starch modifying, or transglycosylating enzymes. The enzymatic hydrolysis is preferred to acid hydrolysis in starch processing industry due to a number of advantages such as specificity of the reaction, stability of the generated products, lower energy requirements and elimination of neutralization steps[30]. Due to the increasing demand for these enzymes in various industries, there is enormous interest in developing enzymes with better properties such as raw starch degrading amylases suitable for industrial applications and their cost effective production techniques[31].

The α-amylase family, *i.e*. the clan GH-H of glycoside hydrolyses, is the largest family of glycoside hydrolases, transferases and isomerases comprising nearly 30 different enzyme specificities[32]. A large variety of enzymes are able to act on starch. These enzymes can be divided basically into four groups: endoamylases, exoamylases, debranching enzymes and transferases[29]:

1. endoamylases: cleave internal α-1,4 bonds resulting in α-anomeric products,
2. exoamylases: cleave α-1,4 or α-1,6 bonds of the external glucose residues resulting in α-or α-anomeric products,
3. debranching enzymes: hydrolyze α-1,6 bonds exclusively leaving long linear polysaccharides, and

4. transferases: cleave α-1,4 glycosidic bond of the donor molecule and transfer part of the donor to a glycosidic acceptor forming a new glycosidic bond.

α-Amylases are ubiquitous enzymes produced by plants, animals and microbes, where they play a dominant role in carbohydrate metabolism. Amylases from plant and microbial sources have been employed for centuries as food additives. Barley amylases have been used in the brewing industry. Fungal amylases have been widely used for the preparation of oriental foods. In spite of the wide distribution of amylases, microbial sources, namely fungal and bacterial amylases, are used for the industrial production due to advantages such as cost effectiveness, consistency, less time and space required for production and ease of process modification and optimization[31].

Among bacteria, *Bacillus* sp. is widely used for thermostable α-amylase production to meet industrial needs. *B. subtilis, B. stearothermophilus, B. licheniformis* and *B. amyloliquefaciens* are known to be good producers of α-amylase and these have been widely used for commercial production of the enzyme for various applications. Similarly, filamentous fungi have been widely used for the production of amylases for centuries. As these moulds are known to be prolific producers of extracellular proteins, they are widely exploited for the production of different enzymes including α-amylase. Fungi belonging to the genus *Aspergillus* have been most commonly employed for the production of α-amylase.

Raw starch digesting amylase

Since many of the commercially available amylases do not withstand industrial reaction conditions, isolation and characterization of novel amylases with desirable properties is very important[33]. From that point of view screening of wild type strains of *Bacillus* sp. is very important. We have found several isolates with promising amylase characteristic[34]. It is important to emphasize that not all of the media used have induced expression of raw starch digesting amylase. Several raw starch digesting alpha amylases which can directly hydrolyze the raw starch in a single step at temperatures below the gelatinization temperature of starch has been reported[35]. Raw starch digesting amylases from *Bacillu*s sp. usually need prolonged time of incubation for efficient raw starch hydrolysis and are not able to digest all types of starch granules with same efficiency[36]. Often, better results were obtained with thermostable raw starch digesting amylases at temperatures between 60 and 70 °C[35]. Recently, amylase from *B. licheniformis* ATCC 9945a was purified and characterized[37]. The advantages of this amylase compared to previously reported ones are related to a high hydrolytic affinity of this enzyme towards different types of raw starch granules; cereals, tubers and roots. Enzyme appears to be a good candidate for the direct hydrolysis of diverse raw starches, using very low doses (0.07 U/mg of starch) and omitting energy intensive and expensive gelatinization step. Raw cereal starches are more completely and rapidly hydrolyzed than those from tubers or roots when digested by single, purified enzymes[38]. Moreover most raw starch digesting alpha amylase reported to date hardly digest potato starch[35,38]. Since corn, wheat and potato are the most important sources of starch in EU[1], enzymes that are capable of digesting all these types of raw starches efficiently are economically attractive.

Conclusion

Starch is a constituent of numerous agricultural feedstocks and a convenient substrate for bioethanol production. However, due to its polysaccharidic composition, it must be hydrolyzed exogenously to microbial cells, in order to be broken down into small sugars, e.g., glucose and maltose. Amylases are thus essential to enable efficient hydrolysis processes. Although processes for the production of bioethanol from starchy feedstocks have been used at large scale for decades, there is a continuous search for technological improvements leading to increases in yield as well as reductions in the costs associated to enzyme production and to the final bioethanol production process itself. Thus, technological challenges are being tackled in a number of fields, such as:

1. Plant biotechnology: The development of grains varieties containing genes for the expression of amylases[39], thus reducing enzyme dose during starch hydrolysis;
2. Microbial molecular biology: Genetic manipulation of strains to obtain strains expressing enzymes for starch hydrolysis[40-42]. Recently, we have produced extracellular recombinant amylase in *E. coli* using DsbA signal peptide sequence approach[43]. Recombinant α-amylase possessed the properties of the native enzyme. Furthermore the recombinant enzyme showed improved thermostability at 90°C and higher efficiency for digesting diverse raw starches comparing to the native enzyme, and comparative ability to hydrolyze raw corn and potato starches as a commercial α-amylase. The properties of the recombinant enzyme suggest the good potential of using this approach for production of fully active industrially important recombinant enzymes.
3. Microbiology: Understanding of metabolic mechanisms and adaptation of microbial cells for tolerance to higher concentrations of ethanol[44];
4. Enzyme technology: Formulation of synergistic enzyme pools for raw starch hydrolysis[45]; production of proteolytic enzymes for the pretreatment of grains, aiming at promoting higher exposure of starch to amylases[24, 46]; and use of protein engineering for the development of enzymes with improved action towards raw substrates[47];
5. Process engineering: Some trends comprise the integration of conversion steps, e.g., simultaneous liquefaction, saccharification and fermentation[47] or simultaneous fermentation and distillation; optimization of process control, by using dynamic strategies[45, 48]; very high gravity fermentation, which contributes to the reduction of capital costs and to the increase of plant throughput[49, 50]; and co-product valorization, through the post-processing and fractionation of DDGS for the separation of higher value-added components and improved use as animal feed.

Acknowledgements

This work was supported by the Serbian Ministry of Education and Science project grant number 172048 and ICGEB research project grant number CRP/YUG11-02.

References

1. Buchholz, K. & Seibel, J. (2008). Industrial carbohydrate biotransformations. *Carbohydr. Res.*, **343,** 1966-1979.
2. Roy, I. & Gupta, M.N. (2004). Hydrolysis of starch by a mixture of glucoamylase and pullulanase entrapped individually in calcium alginate beads. *Enzyme Microb. Technol*., **34,** 26–32.
3. Riley, G. (1850). Improved process in the manufacture of glucose. U.S. Patent 7,148.
4. Brown, J.M., Bemis, W.A., Monroe, F.A.Jr. (1948). .Dextrose syrup. U.S. Patent 2,438,033.
5. Wallerstein, L. & Gray, P. P. (1952). Production of dextrose. U.S. Patent 2,583,451.
6. Hartig, H.P. (1957)**.** Sugars derived from starch. *Food Technol. Aust.*, **9,** 73-83.
7. Honsch, W.M. (1957). Preliminary work for the manufacture of wheat starch glucose. *Starch/ Staerke* **9,** 45-47.
8. Rahman, S. (1995). Food Properties Handbook, CRC Press, Boca Raton.
9. Robertson, G. H., Wong, D.W.S., Lee, C.C., Wagschal, K., Smith, M.R., Orts, W.J. (2006). Native or raw starch digestion: a key step in energy efficient biorefining of grain. *J. Agric. Food. Chem.*, **54,** 353-365.
10. Bawa, N., *et al.,* (2010). Fermentation of glucose and starch particles using an inexpensive medium. *J. Chem. Technol. Biotechnol.,* **85,** 441–446.
11. Fernando, S., *et al.,* (2006). Biorefineries: current status, challenges and future direction. *Energy and Fuels,* **20,** 1727-1737.
12. das Neves, M.A., *et al.,* (2007). State of the Art and Future Trends of Bioethanol Production. *Dynamic Biochemistry, Process Biotechnology and Molecular Biology,* **1(1),** 1-14.
13. Balls, A.K., Schwimmer, S. (1944). Digestion of raw starch. *J. Biol. Chem.,* **156(1),** 203–210.
14. Williams, J. (2006). Break it down now. <http://www.ethanol producer.com/article.jsp?article_ id=319&q=&page=all>. Accessed 30 May 2011.
15. Swanson, T. (2004). Partnering in progress <http://www. ethanolproducer.com/article.jsp?article_ id=804&q=starch%20cold%20hydrolysis&page=all> Accessed 30 May 2011.
16. Galvez, A. (2005). Analyzing cold enzyme starch hydrolysis technology in new ethanol plant design. *Ethanol Prod. Mag.*, **11,** 58–60.
17. Wang, P., *et al.,* (2009). Effects of protease and urea on a granular starch hydrolyzing process for corn ethanol production. *Cereal Chem.,* **86(3),** 319–322.
18. Lehmann, U., & Robin, F. (2007). Slowly digestible starch—its structure and health implications: a review. *Trends Food Sci. Technol.,* **18,** 346–355.
19. Kopnieczny-Janda, G., Li, M., Shetty, J.K., Teunissen, P. (2008). Production of ethanol from barley and DDGS containing reduced beta-glucan and phytic acid. US Patent US2008/003344.
20. Castro, A.M., Castilho, L.R., Freire, D.M.G. (2011). An overview on advances of amylases production and their use in the production of bioethanol by conventional and non-conventional processes. *Biomass Conv. Bioref.,* **1,** 245–255.
21. Huston, A.L. (2008). Biotechnological aspects of cold-adapted enzymes. In: Margesin, R., Schinner, F., Marx, J.C., Gerday, C. (eds). Psychrophiles: from biodiversity to biotechnology. Springer Berlin Verlag, Heidelberg, pp. 347–364.
22. Vieira, B.C. Jr., v (2009). Kinetics of granular starch hydrolysis in corn dry-grind process. *Starch/ Starke,* **61,** 448–456.
23. Kumar, M., *et al.,* (2007). Biocatalytic conversion of granular starch to industrial chemicals. *ACS Symp. Ser.,* **972,** 32–45.

24. Schill, S.R. (2008). CUTC: Researchers continue to study corn utilization <http://www.ethanol-producer.com/article.jsp?article_id=4301&q=starch%20cold%20hydrolysis&category_id=35>. Accessed 30 May 2011.
25. Dufresne, A. (2007). Polymer nanocomposites from biological sources. Biopolymers Technology; Bertolini, A.C. (ed.), Cultura Academica, Sao Paulo, pp. 59-83.
26. Peat, S., Whelan, W.J., Thomas, G.J. (1952). Evidence of multiple branching in waxy maize starch. *J. Chem. Soc.*, 4546–4548.
27. Corre, D.L., Bras, J., Dufresne, A. (2010). Starch Nanoparticles: A Review. *Biomacromolecules,* **11,** 1139–1153.
28. Bordbar, A.K., Omidiyan, K., Hosseinzadeh, R. (2005). Study on interaction of α-amylase from *Bacillus subtilis* with cetyl trimethylammonium bromide. *Colloids Surf. B: Biointerfaces,* **40,** 67–71.
29. van der Maarel, M.J.E.C., *et al.,* (2002). Properties and applications of starch-converting enzymes of the α-amylase family. *J. Biotechnol*., **94,** 137–155.
30. Satyanarayana, T., Rao, J.L.U.M., Ezhilvannan, M. (2005). α-Amylases. In: Enzyme Technology, Pandey, A., Webb, C., Soccol, C.R., Larroche, C. (eds.), Asiatech Publishers Inc., New Delhi, India pp. 189–220.
31. Burhan, A., *et al.,* (2003). Enzymatic properties of a novel thermostable, thermophilic, alkaline and chelator resistant amylase from an alkaliphilic *Bacillus* sp. isolate ANT-6. *Process*. *Biochem*., **38,** 1397–1403.
32. Henrissat, B. (1991). A classification of glycosyl hydrolases based on amino acid sequence similiarities. *Biochem. J.,* **280,** 309–316.
33. Shafiei, M., Ziaee, A.-A., Amoozegar, M.A. (2010). Purification and biochemical characterization of a novel SDS and surfactant stable, raw starch digesting, and halophilic α-amylase from a moderately halophilic bacterium, *Nesterenkonia* sp. strain F, *Proc. Biochem.,* **45 ,** 694–699.
34. {okarda Slavi}, M., Bo`i}, N., Vuj~i}, Z. (2011). Screening a wild type strains of *Bacillus* sp. that produce α-amylases in solid state fermentation. *2nd FCUB ERA Workshop,* P21.
35. Goyal, N., Gupta, J.K., Soni, S.K. (2005). A novel raw starch digesting thermostable α-amylase from *Bacillus* sp. I-3 and its use in the direct hydrolysis of raw potato starch. *Enzyme Microb. Technol.,* **37,** 723–734.
36. Dettori, B.G., Priest, F.G., Stark, J.R., (1992). Hydrolysis of starch granules by the amylase from *Bacillus stearothermophilus* NCA 26, *Process Biochem.,* **27,** 17–21.
37. Bo`i}, N., *et al.,* (2011). Production and properties of the highly efficient raw starch digesting α-amylase from a *Bacillus licheniformis* ATCC 9945a. *Biochem. Eng. J.,* **53,** 203–209.
38. Qates, C.G. (1997). Towards an understanding of starch granule structure and hydrolysis. *Trends Food Sci. Technol.*, **8,** 375–382.
39. Sainz, M.B. (2009). Commercial cellulosic ethanol: the role of plant-expressed enzymes. *In Vitro Cell Dev. Biol. Plant,* **45,** 314–329.
40. Ghang, D.M., *et al.,* (2007). Efficient one-step starch utilization by industrial strains of *Saccharomyces cerevisiae* expressing the glucoamylase and α-amylase genes from *Debaromyces occidentalis*. *Biotechnol, Lett,,* **29,** 1203–1208.
41. Kim, J.H., *et al.,* (2010). Construction of a direct starch-fermenting industrial strain of *Saccharomyces cerevisiae* producing glucoamylase, α-amylase and debranching enzyme. *Biotechnol, Lett.,* **32,** 713–719.

42. Shigechi, H., *et al.,* (2004). Direct production of ethanol from raw corn starch via fermentation by use of a novel surface engineered yeast strain co-displaying glucoamylase and α-amylase. *Appl. Environ. Microbiol.*, **70(8),** 5037–5040.
43. Bo`i}, N. (2012). Hidroliza sirovog skroba - novi pravci. *Hemijski pregled,* **53(2),** 27-31.
44. Hu, X.K., *et al.,* (2007). Genetic dissection of ethanol tolerance in the budding yeast *Saccharomyces cerevisiae. Genetics,* **175,** 1479–1487.
45. Castro, A.M., *et al.,* (2011). Valorization of residual agroindustrial cakes by fungal production of multienzyme complexes and their use in cold hydrolysis of raw starch. *Waste Biomass Valor.,* **2,** 291–302.
46. Vidal, B.C. Jr., *et al.,* (2009). Protease pretreatment to improve ethanol fermentation in modified dry grind corn process. *Cereal Chem.,* **86(3),** 323–328.
47. Vikso-Nielsen, A., *et al.,* (2006). Development of new α-amylases for raw starch hydrolysis. *Biocatal. Biotrans.,* **24(1–2),** 121–127.
48. Murthy, G.S. (2007). Development of a controller for fermentation in the dry grind corn process. PhD Thesis, University of Illinois at Urbana-Champaign.
49. Devantier, R., Pedersen, S., Olsson, L. (2005). Characterization of very high gravity ethanol fermentation of corn mash. Effect of glucoamylase dosage, pre-saccharification and yeast strain. *Appl. Microbiol. Biotechnol*., **68,** 622–629.
50. Pereira, F.B., *et al.,* (2010). Optimization of low-cost medium for very high gravity ethanol fermentations by *Saccharomyces cerevisiae* using statistical experiments design. *Bioresour. Technol*., **101,** 7856–7863.

Harnessing the Biological Activity of Natural Products: Isolation, Synthesis and Screening of Biological/ Pharmacological activity

Niko S. Radulović*

* *Department of Chemistry, Faculty of Science and Mathematics, University of Niš, Niš, Serbia*
e-mail: nikoradulovic@yahoo.com

Plant secondary metabolites have been a constant inspiration in the discovery and development of new biologically and pharmacologically active compounds. The natural pool of molecules possessing antimicrobial, cytotoxic, analgesic and other properties is a consequence of their crucial role in the non-stop evolutionary selection enabling plant species to survive the harsh environmental conditions. Harnessing the activity of these natural products requires detailed structural elucidation and a means, either through isolation or synthesis, of acquiring an amount sufficient to perform biological assays. In the lines that follow I will try to review the basic up to date knowledge on the subject of natural product in drug discovery and try to confer the rational behind it, whereas the oral presentation of this invited lecture will provide an overview of our work performed in the field of natural products chemistry aiming at finding new and more efficient antimicrobial, antinociceptive, antianxiety, antioxidant and/or hepatoprotective principles of enthopharmacologically renowned plant species from Serbia and around the world. The work in our lab on the analysis of volatiles of over 100 plant species belonging mostly to the Asteraceae, Apiaceae, Rutaceae and Brassicaceae plant families allowed the identification of more that 1000 different compounds. These essential oils and other solvent extracts were fractioned in a bioassay guided manner and yielded their active principles in pure state. Additionally, gram quantities of some selected plant metabolites were made available through synthesis and permitted a more in depth testing of their pharmacological activity.

Introduction

Of the 520 new pharmaceuticals approved between 1983 and 1994, 39% were derived from natural products, the proportion of antibacterials and anticancer agents of which was over 60%[1]. Between 1990 and 2000, a total of 41 drugs derived from natural products were launched on the market by major pharmaceutical companies, including azithromycin, orlistat, paclitaxel, sirolimus (rapamycin), Synercid, tacrolimus, and topotecan. In 2000, one-half of the top-selling pharmaceuticals were derived from natural products, having com-

bined sales of more than US $40 billion. These included the biggest selling anticancer drug paclitaxel, the "statin" family of hypolipidemics, and the immunosuppressant cyclosporin. During 2001 we have seen the launch of caspofungin from Merck and galantamine from Johnson & Johnson, with rosuvastatin, telithromycin, daptomycin, and ecteinascidin-743 due to follow in 2002.

Despite the figures, the popularity of natural products, particularly those from higher plants as leads for new pharmaceuticals, tends to fluctuate. At the time of writing, several of the world's biggest pharmaceutical companies have reined back their natural product drug discovery programs and have placed great faith in combinatorial chemistry, coupled to very high throughput screening. Time will tell whether this is a wise stratagem, or whether the unique features of compounds that are themselves derived from living organisms will once again see renewed acceptance.

The abundance of plant and microbial secondary metabolites and their value in medicine are undisputed, but one question that is only partly answered concerns the reasons for this abundance of complex chemical substances. In the past, the production of what we would now call "bioactive" substances was a mystery. A modern view is that these compounds have a role in protecting the otherwise defenseless, stationary plant from attack by mammals, insects, fungi, bacteria, and viruses. Taking morphine as an example of a secondary metabolite whose value to the plant is not entirely obvious, 14 steps are required from available amino acids, including at least one step that is highly substrate specific[2]. The presence of morphine in the tissues of *Papaver somniferum* must therefore confer a selectional advantage on the plant[3]: genetic code is required for each of the enzymes involved in the biosynthesis, valuable amino acids are utilized in forming the enzymes, and a relatively scarce nutrient (nitrogen) is locked up in the compounds produced. If the morphine did not continue to have value for the plant, mutants would have arisen with the advantage of not having a drain on their metabolic resources.

We can only guess at the ecological functions of morphine. Perhaps a mammalian herbivore that consumed too many poppies would become drowsy and itself fall prey to a carnivore. It may be significant that the cannabinoids, produced in greatest abundance in the nutritious growing tips of the plant, also induce mental effects that would compromise a herbivore's ability to escape a predator. Whatever their natural protective functions, natural products are a rich source of biologically active compounds that have arisen as the result of natural selection, over perhaps 300 million years. The challenge to the medicinal chemist is to exploit this unique chemical diversity. The following account illustrates how natural products can be or have been used as what are called *lead compounds*, or templates for the development of important medicines.

Attributes of Natural Products

It is now well established that natural products have chemical diversity (see Table 1 for the structures of a number of selected plant secondary metabolites) and biochemical specificity, which clearly differentiate them from synthetic and combinatorial compounds and that make them favorable lead structures for drug discovery[4]. Natural products contain greater

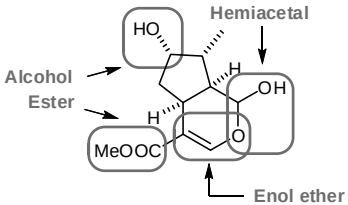
chirality and steric complexity than either synthetic drugs or combinatorial libraries, and while synthetic molecules contain significantly higher numbers of nitrogen, sulfur, and halogen containing groups, natural products bear higher numbers of oxygen atoms[5,6]. Natural products can be differentiated from trade drugs or other synthetic molecular libraries on the basis of scaffold architecture and pharmacophoric properties[7]. They differ significantly from synthetic drugs and combinatorial libraries in the ratio of aromatic ring atoms to total heavy atoms (lower), number of solvated hydrogen bond donors and acceptors (higher), and by greater molecular rigidity[8], and they have a broader distribution of molecular properties such as molecular weight, log *P* and diversity of ring systems. Indeed, Grabowski[9] reported more than 100 scaffolds in a limited set of natural products that were not contained in any other compound set.

It is often said that natural products lack suitable drug-like properties or are structurally too complex for efficient postscreen hit-to-lead development. Detailed analysis, however, shows that high-quality natural product libraries compare quite favorably in terms of drug-like properties. Feher and Schmidt[6] examined representative combinatorial, synthetic, and natural product compound libraries on the basis of molecular diversity and "drug-likedness" properties such as molecular weight, number of chiral centers, molecular flexibility as measured by number of rotatable bonds and ring topology, distribution of heavy atoms, and Lipinski-type descriptors. In the overall picture for drug-like properties, Schneider and Lee[7] determined that the fraction of natural product structures with two or more "rule-of-five" violations is equal to that of trade drugs, approximately 10%. By applying suitable selection criteria, it is possible to construct diverse pure natural product libraries containing a high proportion of molecules with good drug-like properties[10]. Indeed, it is now deemed important to incorporate a certain degree of "natural product-likedness" into any screening library[11]. If properly characterized and constructed, a purified natural products library need not be large to be an effective screening source of bioactive molecules[12].

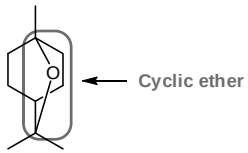
Natural Products Libraries Complexity: Mixtures to Single Components

The success of any screening campaign is dependent on the nature and quality of the screening library. Natural product libraries may be composed of crude or complete extracts (tens to hundreds of compounds per sample), semipurified mixtures (roughly 3–10 compounds per sample) or pure natural products. Crude extract libraries are generally made by liquid- or solid-phase extraction of the fermentation broth or plant material. They require only modest sample preparation but demand the most resources for the identification and isolation of the bioactive constituents. In crude natural products libraries, highly polar or highly lipophilic components of the crude extract may interfere with the functioning of the assay, causing false positives or false negatives. In addition, one or more rounds of chemical purification and biological assay may be necessary to identify and isolate the active component(s) from the extract. This requires the continued availability of assay resources to support the isolation and purification along with additional time to resolve the hit and furnish pure compound for further biological evaluation.

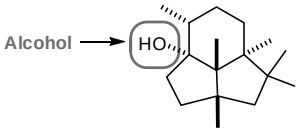
Table 1. Some selected plant secondary metabolites: insight into the diversity of skeletal types, functional groups and substitution patterns



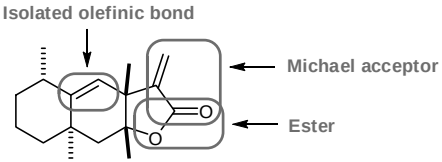
Loganetin (monoterpene-iridoid)



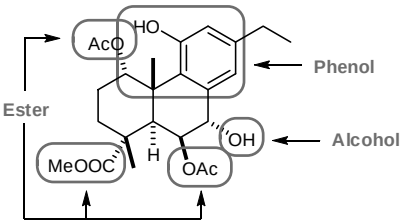
1,8-Cineole (Monoterpene)



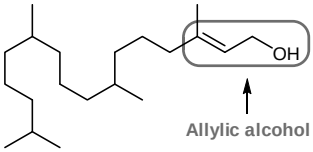
***epi*-Presilphiperfolan-1-ol** (Sesquiterpene)



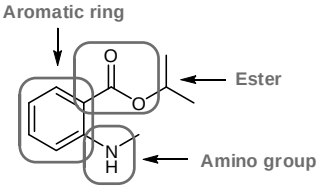
Alantolactone (Sesquiterpene lactone)



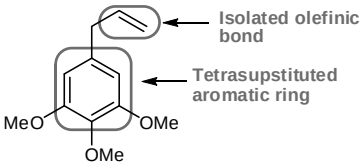
Eurabienol (Diterpene)



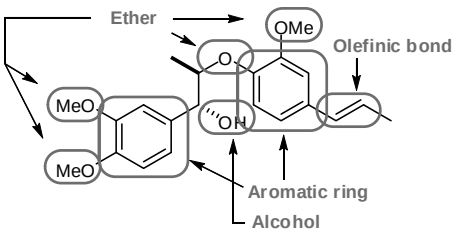
(*E*)-Phytol (Diterpene)



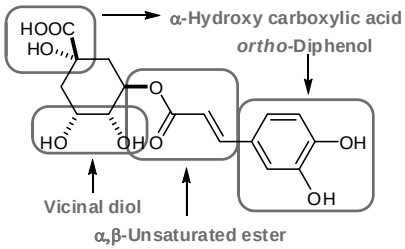
Ternanthranin (C_6C_1 Shikimate metabolite)



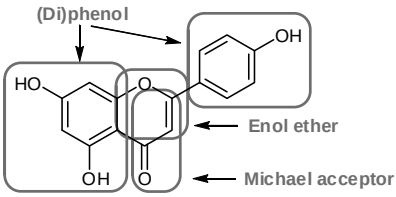
Elemicine (Phenylpropanoid)



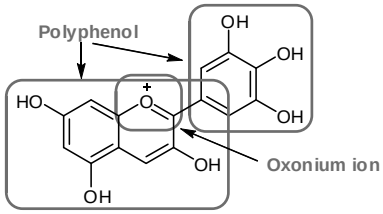
Virolin (Lignan)



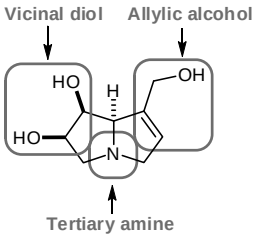
Chlorogenic acid (Phenylpropanoid)



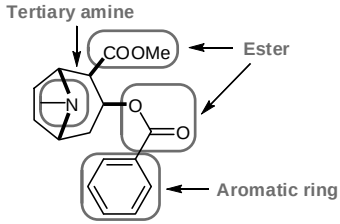
Apigenin (Flavone)



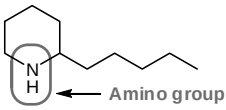
Delphinidin (Anthocyanin)



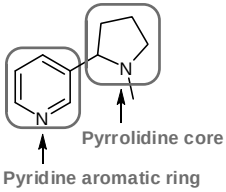
Crotanecine (Pyrrolizidine alkaloid)



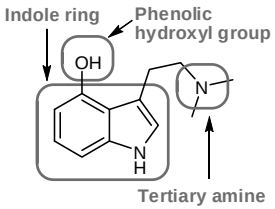
Cocaine (Tropane alkaloid)



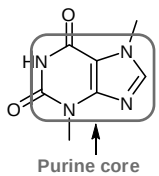
Conmaculatin (Piperidine alkaloid)



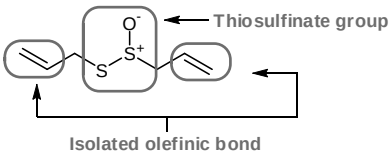
Nicotine (Pyridine alkaloid)



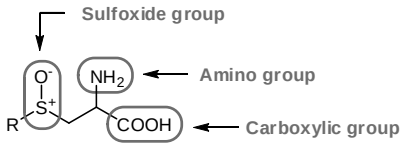
Bufotenin (Indole alkaloid)



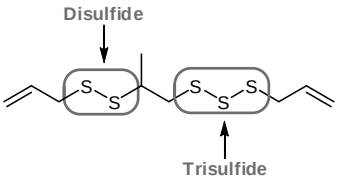
Theobromine (Xanthine i.e. purine alkaloid)



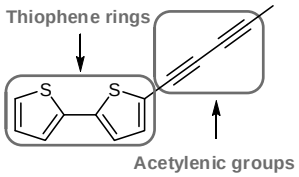
Allicin



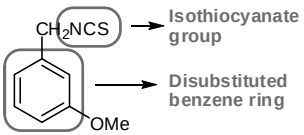
***S*-alk(en)yl cysteine sulfoxides**



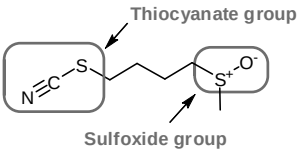
8-Methyl-4,5,6,9,10-pentathiatrideca-1,12-diene



5-(Penta-1,3-diynyl)-2,2'-bithiophene
(Polyacetylene)



3-Methoxybenzyl isothiocyanate



1-(Methylsulfinyl)-4-thiocyanatobutane

Prefractionated libraries can be an effective strategy to alleviate interferences encountered with crude libraries, and may also shorten the time needed to identify the active principle. There are many variations of this approach and each offers advantages of expediency or purity gained at the cost of up-front partial purification[13].

Samples produced by the prefractionation approach are simpler mixtures and the final resolution of active components requires fewer purification steps. Interferences are reduced due to the fact that extremely polar and extremely nonpolar components are separated from the bulk of the library samples[14]. Moreover, the relative concentration of minor components is increased over that in the crude, thereby enhancing the opportunity to uncover novel biologically active metabolites. The advantages of the prefractionation approach need to be balanced against the resource investment necessary to select, prepare, characterize, and maintain such a partially purified natural product library. Since it creates several samples from a single extract, prefractionation increases the size (and cost) of the library for a given number of extracts. Given the often-substantial costs associated with assay reagents, especially against high-value targets, it is essential that redundancy in screening libraries be minimized. This entails analytical characterization of the natural product library contents in the form of HPLC–MS or other techniques, to assure a minimum of redundancy and a maximum of chemical diversity.

Purified natural product libraries offer the advantage that the hit detection process is similar to that of synthetic single component libraries, and the robustness of the hit identification process depends primarily on the purity and chemical integrity of the library itself. Purified libraries offer considerable advantage in the detection of quality hits and in moving forward immediately, since the bioactive principle requires no isolation from a mixture. The downside to this approach is that even with modern methods of separation and automation, substantial resources are required to prepare pure natural product libraries and trace components will not be fully captured in pure form[15]. However, the increasing migration of the industry toward precision ultrahigh-throughput screening coupled with the overall accelerated pace of drug discovery have prompted a move toward highly processed or even pure natural product libraries[16]. Advances in automated separations and sample processing have made purified natural products libraries much more achievable[17]. Besides familiar separation methods such as reversed phase HPLC, newer applications of countercurrent and super critical fluid extraction and chromatography are finding use in natural products library construction[18,19].

In any library strategy, it is important to consider two points: (1) it is essential to accurately characterize the content of the library to minimize duplication and maximize the chemical diversity and integrity[20] and (2) maintaining the purity and integrity of a sizable pure compound library is an often-underestimated technical challenge[21]. This is especially true for pure natural products libraries that contain complex unidentified molecules.

Screening for natural product activity

What is a screen? A screen is an assay or biological assay that provides a tool that can be used to test for or establish the presence and level of a target activity in a specific sample. Bioassays in a screening program should be rapid, simple to conduct, relevant, capable of being automated, cost effective, and of the potential to deliver high throughput[22-24]. Appropriate technology should be used to permit low limits of detection. This last point is important because the concentration of desirable compounds is unknown in each sample and so it behooves one to strive for the lowest possible limit of detection. Screens should also be specific for the molecular or cellular therapeutic target of choice. Appropriate additional discriminatory tests outside of the focus of the chosen target activity, such as cytotoxicity measurement for cell-based assays, or isotype specificity tests for molecular assays, are valuable in that they provide additional information relative to the overall value of a potential hit. Furthermore, data generated from all screens in which samples are tested should be compared so that selective hits can be identified at an early rather than a late stage. Such a combination of specific screens, data comparisons, and discriminatory assays makes possible the earliest selection of the best hits for continued work and success. The screens must work in the presence of the compounds to be tested and accordingly must be compatible with a given molecule's physico-chemical characteristics. Accordingly, natural product screens need to be operational in the presence of solvents and buffered against extremes of pH and ionic strength and should not be affected by the presence of color. Screens should always incorporate the proper use of controls, both positive and negative. Screens should be bidirectional with regard to their output and have a defined and easily interpretable endpoint. Screens need to be capable of delivering quantifiable data. Screens can be designed in such a fashion as to monitor only a single biological activity. However, there is value to the approach of coupling biological screens to evaluate multiple general biological activities in addition to the target activity[25]. This is because a compound that does not provide a target hit may well generate value for itself by demonstrating some other unrelated and unexpected activity. Alternatively, compounds that do provide hits may well expand their value by demonstrating other unexpected types of biological activity. Additional considerations of the construction of screens, screening programs, and screen design are beyond the scope of this review, and the reader is instead referred to an excellent reference on the subject by Gad[26]. What will be addressed here are some of the common features of screens and screening specific to research on the development of natural products as drugs. Before one can begin to screen, it is important to know for what one wants to screen. Drug discovery begins with basic ideas, ideas relevant to therapeutic targets and sources of compounds[27-29]. Therapeutic targets can arise from genomics, the molecular cloning of receptors and signaling molecules, a detailed understanding of physiology, biochemistry, and pathology, research into folklore or ethnomedicine, and knowledge of the traditional uses of natural products. Sources of compounds can present from existing chemical libraries, historical compound collections, natural product libraries, combinatorial libraries, rational chemical synthesis, general or targeted literature searches based upon existing knowledge or leads, and antisense oligonucle-

otides. The simultaneous consideration of all or at least several of these areas is essential to the original design of a screening program. A totally random approach in the selection of a source can be coupled with mass screening, but such a path is typically not successful. But to be fair, the random approach is more likely to generate compound novelty[30]. Generally, approaches utilizing literature searching, existing chemical libraries and folklore, ethnomedicine, or traditional medicine are the most popular because of their cost effectiveness. It is worth noting that programs and selections based on or incorporating ethnomedicine or folklore are five times more effective in the ultimate generation of leads. The most effective approach is generally considered to be a mixing of as many components of the therapeutic arm with the compound source arm as opposed to the selection of any single aspect. It is worth noting at this point that a new approach is being used in the field of natural products that leads to the generation of "unnatural natural product compounds"[27]. This approach is termed *biochemical combinatorial chemistry*. In this technique, appropriate secondary metabolic enzymes are isolated from a crude natural product mixture. These enzymes are then used to generate unnatural metabolites, which are isolated and then subjected to a bioassay procedure that couples a bioassay to an analytical procedure permitting structure elucidation. What does one put into a screen? It is very tempting to consider the purification of natural products into their individual components before embarking on screening activities. However, this is a very economically challenging and financially unrealistic approach[31]. The classical approach is to design a screen or screening program that will permit the use of the assay or assays to provide guidance to successive steps in purification. An advantage of this is that the effectiveness and efficiency of purification can also be simultaneously evaluated at the same time as biological activity is being enhanced. Ultimately, after sufficient purification, a chemical structure can be determined for the active moiety. However, Grabley and Sattler[32] are of the opinion that the use of cost-effective physico-chemical and chemical screening procedures will facilitate biological screening because of an ability to provide purer extracts if not pure compounds for biological screening. Some objectives should be kept in mind when preparing natural product extracts for their ultimate introduction into the screening process[33]. First, every attempt should be made to stop ongoing biological processes. Second, steps should be taken to provide chemical stability of the compounds in the extract. Third, efforts need to be made to minimize losses of material. Fourth, sample preparation costs need to be minimized. When trudging through the iterations required to purify mixtures, one should be sure to: (1) focus on the activity of interest, (2) focus on the compound(s) of interest, (3) eliminate nuisance materials such as cell parts, biopolymers, and other compounds not of interest, and (4) be sure to enrich the composition of compounds of interest. What types of screens, assays, or bioassays should be used? Assays for activity can be performed at a variety of levels ranging from the molecular level to the whole organism. While it is true that the high-throughput screening of synthetic compounds generated by combinatorial methods may be best achieved at the molecular level, Duke et al.[27] are of the opinion that natural product screening should be performed at the highest level possible since more effort per compound has been invested in the discovery of each compound. His-

torically, natural products have been subjected to what is termed bioassay- directed isolation. In this approach, a crude natural product mixture is subjected to fractionation and the individual fractions then bioassayed for specific biological activity. This process continues repetitively with comparison of individual fraction assay data to a bioassay database. When the data is shown to match a previously known profile, the process is terminated and the sample is discarded. If the data is shown to provide a new profile, the structure of the compound is determined. However, this approach can lead to the rediscovery of previously known compounds after significant effort has been expended. Low-molecular-weight natural products from a variety of sources represent unique structural diversity. In order to more adequately and efficiently access this diversity, various new strategies improving targeting and direction have been developed[32]. Modern separation/chemical characterization approaches can eliminate much of this problem by identification of the compounds before they are subjected to bioassay. Indeed the coupling of such techniques to biological screens can improve the quality of the assay result and shorten research and development time frames. These new tandem approaches are termed fractionation-driven bioassays. While biological screening directly correlates to a predefined biological effect, physico-chemical and chemical screens do not. In this latter case, lead selection is based on either physico-chemical properties or chemical reactivities. In both cases, the first step is the chromatographic separation of compounds from the complex source mixture. In the second step, the physico-chemical properties or chemical reactivities of the separated compounds are analyzed. Both of these chemical-based strategies have proven to be of value as auxiliary or supplemental methods to biological approaches. Data generated from physico-chemical and/or chemical screening is very helpful in the de-replication or early identification and exclusion of known or otherwise unsuitable compounds that occur during high-throughput biological screening programs. Furthermore, the use of physico-chemical and chemical screening will aid in the establishment and building of natural-product-based compound libraries, which could then be used more broadly in testing programs. There are two general types of new tandem assays. The first of these is referred to as the fractionation-driven bioassay. In this method, a crude natural product mixture is subjected to fractionation and the individual fractions then subjected to nuclear magnetic resonance (NMR) spectroscopy or mass spectrometry/ mass spectrometry (MS/MS). The structures of the compounds in the individual fractions are identified; and, if they are known, their biological activity profiles are evaluated from an existing database. If the structures are unknown, then the compounds are subjected to bioassay. In an alternative approach, termed the isolate and assay approach, a crude natural product mixture is subjected to automated fractionation and purification. The individual fractions are then subjected to bioassay. Desirable biological activity serves as the trigger to subject the sample to NMR or MS/MS and ascertain the structure(s). If the material is a novel compound, the structure can be optimized. If the material is a previously known compound, it may well be discarded, depending on its biological activity or toxicological profile. A broad range of screening technologies are currently available for use in screening for natural-product-based drugs[24]. For molecular targets, such procedures would include gener-

alized solution-phase assays, immobilized substrate assays, scintillation proximity assays, and time-resolved fluorescence assays. For cell based targets, cell signaling, cell communication, cell receptor, and reported gene assays are available. Isolated subcellular systems are also available[34]. Bioassays can, if desired, incorporate lower level organisms, isolated vertebrate organs, or whole animals. In short, systems are limited only by the creativity and design of the screener. Examples of screening programs can be readily found in the literature. Just to reference a few, Quinn[35,36] has reported on his efforts on prospecting the biodiversity available in Queensland; Mehta and Pezzuto[37] have published on their program to identify cancer preventive agents from plants; El Sayed[30] has reported on his screening program for antiviral agents; Barrett[38] has written on his program to find novel antifungal agents; Yang and co-workers[39] has reported on his search for anti-HIV compounds from natural sources; and Bindseil and co-workers[15] have published on their experiences on screening with pure compounds. The emergence of high-throughput screening (HTS) has permitted the rapid screening of extremely large collections of structurally diverse synthetic compounds against a variety of novel and diverse disease targets[35,36,40,41]. However, despite initial hopes that HTS was the final solution for drug discovery, for reasons that will not be discussed here, HTS has not achieved that distinction. Nevertheless, HTS is still a powerful tool. HTS strategies focus on the ability to screen large libraries of compounds. However, the limiting factor in HTS is the ability to access large numbers of chemically diverse substances. Natural products are undoubtedly the greatest source of structural diversity. Accordingly, HTS of the unparalleled diversity that exists in natural product extracts offers the highest probability for discovery of novel lead compounds and should therefore be viewed as being complementary to compounds generated from combinatorial chemistry alone. The synergistic melding of HTS and natural products has started, and, as it progresses along its development path, exciting new breakthroughs will undoubtedly be presented. As a compound generates interest through a variety of screening assays and progresses down the drug discovery path, certain questions need to be asked[38]. Is the chemical structure of the compound novel? Is the mechanism of action of the moiety novel or of utility? Is the biological activity of the compound useful? Is the potency of the compound reasonable? Is a proof of concept available? Is chemical modification of the structure possible? Is solubility a problem? Can the material be synthesized on a large scale? The ability to ask and answer these questions effectively early on in the process will be highly predictive of the ultimate success of a particular line of research. While there are no “correct” answers to these questions, as answers will be different depending on the therapeutic indication and other available therapeutic alternatives, they still need to be addressed to provide proper program focus. Before ending this section, it is important to emphasize the necessity and importance of keeping detailed records as one initiates the screening process[33]. Maintenance of a secure physical inventory with a controlled environment and adequate records is another important detail. The use of bar code identifiers is very desirable for samples and relevant computer programs are readily available. It should be considered to be essential to establish a complete database, which should include the source, source location, isolation details, any relevant taxonomic information (kingdom,

genus, and species), and any other relevant information (third-party suppliers of reagents, potential pathogenicity or toxicity, relevance to any international biodiversity treaties), preservation methods, age, position or location within a freezer, and the like. Other considerations in the design and management of a successful screening program could be limitation of access, storage of reference or backup samples, storage of a backup copy of inventory and database, establishment of tracking procedures, writing of standard operating practices (SOP) for sample handling, and temperature alarms.

Isolation and purification of natural products

Why do scientists working with natural products isolate and purify them? For one of two reasons: (1) to ascertain what the natural product is and (2) to carry out sufficient experimental work necessary to biologically characterize or profile the compound. It can be quite a sobering experience to look at a flask full of dark-colored, inhomogeneous sludge and liquid and realize that one is going to attempt to isolate just one particular type of molecule from all of the other materials that are present. To put this in perspective, typically the material sought after represents only about 0.0001 percent of the total biomass in the flask[22,23]. Then, just to make things even more challenging, the desired molecules can also be bound with other materials and molecules present in the mixture, making the desired compound(s) even harder to purify. It is important to keep in mind that the isolation of natural products differs from that of the more prevalent biological macromolecules. This is because natural products are typically secondary metabolites and as such are smaller in size, chemically more diverse in structure, and present in smaller concentrations than the more homogeneous proteins, carbohydrates, lipids, nucleic acids, and the like. This section will not present a condensed work on separation science and procedures as that is best left to any of the myriad analytical chemistry textbooks. What will be attempted here is to provide sufficient guidance on the isolation and purification of natural products so that proper focus can be assured in the design and implementation of a successful isolation and purification program. Before initiating an isolation and purification, there are a number of basic questions that need to be asked and answered. First, what are you trying to isolate and purify? There are a number of different possible targets: (1) an unknown compound associated with a particular biological activity, (2) a previously known compound present in a specific organism, (3) a group of compounds within an organism that are all structurally related to each other, (4) all of the metabolites produced by one natural product source that are not produced by another closely related source, or (5) all of the molecules of a particular organism. Second, why are you trying to isolate this material? While the asking of such a question might appear to be superficially inane, it is important to know why you want something so that you know how much of it you might need. Possible reasons for carrying out an extraction might be: (1) the generation and supply of larger amounts of an already known compound so that more extensive biological testing such as pharmacology and toxicology can be performed on the material, (2) the purification of a small amount of material for initial biological and chemical characterization to be performed, or (3) to purify sufficient material in order to conduct complete structural

studies and further biological activity characterization. Third, what type of purity is desired? If a natural product compound is to be used for biological testing, it is important to know not only the degree of purity of the material but also the nature of the impurities. It needs to be appreciated that the impurities themselves can contribute significantly to any biological activity observed in the screening program. If the material is to be used in more refined pharmacological or pharmacokinetic testing, then the material should generally be at least 99 percent pure. If, on the other hand, the material is to be used only for chemical characterization, the acceptable level of purity can range from 95 to 99 percent. Such a range of purity will generally be sufficient for the determination and assignation of a complex chemical structure via such techniques as NMR spectroscopy, infrared (IR) spectroscopy, and MS/MS spectrometry. It should be noted that if the compound under consideration is present in a high concentration in the starting material and a standard for that compound already exists, then structural confirmation can be achieved with less pure material and the associated purification scheme will be composed of fewer steps. Depending upon one's goals, varying degrees of purification may be acceptable. X-ray crystallographic studies will demand material of 99.9 percent purity, while detection of the presence or absence of a specific structural feature via analysis of the ultraviolet spectrum may tolerate purities down to a level of 50 percent. An important concept of purification is that the relationship between purity of compound achieved during natural product extraction and the amount of effort expended to achieve such a level of purity is almost exponential in nature. When starting with a crude, complex mixture, it is very easy to eliminate large components of unwanted material. However, as the purity begins to escalate, it can become infinitely more challenging to improve purity levels. For example, the effort required to go from 50 percent purity to 90 percent purity can pale in comparison to the effort required to go from 99.5 to 99.9 percent purity. In concordance with this, it is fair to state that the relationship between purity level and yield are also exponentially related. In a purification scheme no step delivers the desired material in 100 percent yield. Each extraction step results in the loss of material, and when working to attain very high levels of purity, losses can be extreme. While it may be necessary to take only very "centralized" cuts in a purification step, keep in mind that the "tail" cuts can themselves be later subjected to reprocessing. Fourth, what type of fractionation should be used in the isolation and purification scheme? All separation processes involve the division of a mixture into a number of discrete fractions. This process is called fractionation. Such fractions can be physically separate such as the two phases of a liquid–liquid extraction or they may not be physically separate such as the continuous eluate from a chromatography column. The eluate from a chromatography column can then be artificially divided into fractions via the use of a fraction collector. The method of fractionation depends on the sample and the goals of the separation. Fractions are typically equal in size and can be large or small in volume. The collection of a large number of small fractions improves the probability that each fraction might contain a pure compound. However, such an approach requires significant work in the analysis of each fraction. Additionally, this approach may spread the desired compound over so many fractions that if the target molecule(s) was present originally only

in low levels, it may prove undetectable in any one of the fractions. Alternatively, if the separation process is cruder, employing the collection of only a few large volume fractions, a more rapid and facile tracking of the desired compound and its activity is possible. Fifth, what is the nature of the compound? The answer to this question depends on how much is already known about the compound. General features that are useful at this stage of the project are acid/base properties (p*K*a, p*K*b), molecular charge, stability, and solubility (hydrophilicity/lipophilicity). If the target molecule is an unknown moiety, it is very likely that little of this information is known and all of it will have to be determined along the way. If one is isolating a known compound, much of this information will already be available. Finally, if the goal is to isolate a number of secondary metabolites rather than a single molecule, then the value of this step is less important, but an appreciation of the relative values of these parameters can still be useful with regard to understanding the characteristics of the mixture. Sixth, where is the desired activity localized? Each potential source of a natural product source—whether it is plant, tree, moss, bacteria, vertebrate, invertebrate, insect, terrestrial, or marine based—has components or parts in which the desired activity or compound is present in greater concentration as opposed to other parts in which the compound is present in lesser amounts. To obviate any problems associated with dilution of the compound and its activity, the initial biomass should be selected on the basis of its content of the target biological activity. Only with the thoughtful provision of answers to the above questions can one have a clear idea of what one is attempting to achieve and how to successfully secure the project goals. It should be obvious that there is no correct or incorrect protocol or standard operating procedure for the isolation and purification of natural products. Indeed, the final method or scheme itself is most likely to vary with the answers to the above questions as well as the natural product source and the specifics of the assays and biological assays that are to be used in the screening program. However, a consult of the literature is essential during the design and construction of an isolation and purification program for any natural product compound[42,43]. While it is possible that extensive data may have already been published on the compound one is trying to isolate or compounds related to it, it is also entirely possible that nothing is known. Regardless, proper use of the natural products literature can facilitate the effort invested into the design and implementation of a specific isolation and purification program. A variety of different techniques can be used for the isolation and purification of natural product compounds[22,23,44]. These techniques include, but are not limited to, solid-phase extraction[22,23], high-performance liquid chromatography (HPLC)[22,23], gradient high-performance liquid chromatography[22,23], bioautography[43], thin-layer chromatography (TLC)[22,23], countercurrent chromatography[45-47], droplet countercurrent chromatography[48], vacuum column chromatography[43], desalting[42], liquid–liquid chromatography[49], paper chromatography[22,23], ion exchange chromatography[22,23], size exclusion chromatography[50], affinity chromatography[22,23], acid–base switching technology[51], centrifugal partition chromatography[48], liquid–solid chromatography[22,23], microwave-assisted extraction[52], pressurized solvent extraction[52], large-scale solvent extraction[51], and supercritical fluid extraction[53,54]. While the theories along with the relative advantages and disadvantages be-

hind each one of these procedures have not been discussed here, the listing will serve as a catalog of potential techniques available to the researcher. Specific details on any of these procedures can be obtained from any number of books on separation. A debate still exists as to the timing of isolation and purification in the drug discovery process[31]. It is always tempting to isolate and purify before screening, but understandably this can present a challenge. Classical approaches have used a successful marriage between purification steps and bioassay activity assessment to isolate, identify, and fully characterize natural product compounds. It should be appreciated that with the advances in chromatographic and analytical techniques that have taken place over the last 15 years, the time required to proceed from an initial hit to an identified active compound should take no longer than for the resynthesis and purification of a potential active compound from a combinatorial library. Accordingly, the timing of isolation and purification in the natural product drug research and development timeline should not persist as such a point of contention as previously[15].

Structure elucidation of natural products

The chemical structures of natural product compounds are tremendously diverse and can be very elegant in their nature[55-58]. Such diversity can present a challenge to the analytical or medicinal chemist attempting to unravel the mystery of the chemical structure of an unknown material presented to him or her. However, modern technology has made structure identification simpler and faster. Today, scientists take for granted such techniques as MS, MS/MS, IR, Fourier transform infrared spectroscopy (FTIR), NMR, Fourier transform nuclear magnetic resonance spectroscopy (FTNMR), and others. It is beyond the scope of this review to discuss the theory and relative merits of each of these techniques, and the reader is urged to consult appropriate textbooks on analytical chemistry. However, it is worth mentioning that some particularly exciting developments in structure determination pertinent to the area of natural products have come from the field of computer- assisted structure elucidation (CASE). Several computer programs have become available for scientists to use and a number of publications reporting on the utility of this technological advancement have been forthcoming[59]. As has been previously described, the elucidation of the structure of a natural product begins with the collection of a crude material. This material is then subjected to a series of separation steps, usually involving chromatography, delivering in the end pure compound(s). Finally, a set of spectroscopic and spectrometric experiments are performed on the pure compound to delineate the structural characteristics. Such analysis may even reveal the twodimensional or three-dimensional structure of the isolated chemical. Time is money in the drug development business and in order to accelerate activities, the following steps of the structural elucidation process should be considered to be targets amenable to automation: (1) the choice of the smallest group of procedures that is most likely to reveal the unknown structure [NMR, FTNMR, MS, MS/MS, IR, FTIR, ultraviolet (UV), etc.], (2) the acquisition of data from the selected procedures, (3) the analysis of data from the selected procedures, and (4) the use of a computer program to construct the structure from collected spectroscopic and spectrometric data. Historically, structure identification has occurred after

purification was complete. However, not infrequently in these cases, structural identification revealed that all of the previous laborious steps of purification had produced a compound that was already known or of an undesirable type. Now, the coupling of liquid chromatography (LC) or high-pressure liquid chromatography (HPLC) with such technologies as MS, MS/MS, and NMR has permitted the construction of devices that allow the injection of a crude sample, separation of the sample using automatically determined optimized conditions, and on the fly spectrometry or spectroscopy followed by CASE for each set of acquired spectra. The identification of unwanted compounds or de-replication should occur as early as possible in the natural product isolation and purification process to avoid the loss of time and funds. The development of coupled techniques has permitted the achievement of that goal. Indeed, coupled techniques such as LC/NMR/MS have now evolved and have potent application in the pharmaceutical field. As the sensitivity of instrumentation continues to improve, the value of these coupled techniques will increase even more. A number of powerful aids for NMR spectroscopic interpretation have emerged and are now readily available from instrument manufacturers and include programs such as Auralia and AMIX. However, any comprehensive CASE program will be based on a quality structure generator. To this point, only a few high-quality, pure structure generators have been developed. While over the years there have been a variety of programs that have been developed, they have been limited to those researched and developed by small, private groups. Now there are some highly capable programs that are commercially available, such as Assemble (Upstream Solutions GmbH, Zurich, Switzerland) and MOLGEN (http://www.molgen.de). A well known deterministic CASE system that is often cited in the literature is CISOC-SES, which is now commercially available under the acronym NMRSAMS (Spectrum Research, Madison, WI, USA). Another deterministic CASE program, COCON has been relatively recently introduced, and several examples demonstrating its value in structural elucidation have been reported[59,60]. Deterministic algorithms have a limit to the size of the molecule with which they can work. To overcome this, a stochastic structure generator has been published for the computer-assisted structure elucidation of organic molecules[61]. The name of the program is SENECA. This program is written in Java and therefore is platform independent and allows for a simple plug-in mechanism for new spectroscopic data types. Theoretically, many different kinds of properties can be plugged into this system, as long as the property can be reliably calculated from a generated molecule. Classical CASE systems can at least attempt to provide the two-dimensional structure of an unknown molecule. Now, with the greater exposure of, availability of, capabilities of, and demand for CASE programs, efforts are being made toward incorporation of the ability to confidently determine the three-dimensional structure of unknown molecules[62]. Advancements in chromatography, spectrometry, and spectroscopy together with breakthroughs in the coupling of these technologies are important steps in the production of a fully automated and integrated natural products structure determination instrument, which will provide significant advantage to the early, rapid, and facile identification of new natural-product based drug opportunities.

Synthesis of natural products

Once a natural product compound has been screened for biological activity, isolated, purified, its structure identified, and the pharmacological profile refined, the journey is not over. The molecule may turn out to be too complex in nature and too expensive to be synthesized. Indeed, when compared to a purely man-made synthetic alternative, many times the natural product compound is quickly eliminated from further consideration because of considerations of time and potential costs of synthetic production. Any given natural product compound may possess unacceptable physicochemical, pharmacodynamic, pharmacokinetic, or bioavailability properties or demonstrate excessive toxicity and will therefore require optimization of its chemical structure. Optimization involves a dissection of the lead molecule and the synthetic addition, removal, replacement, or modification of substituent groups so as to enhance the utility and efficacy of the molecule. The synthesis of a complicated molecule is a very difficult task since every group and atom must be placed in a proper position and with the correct stereochemistry. Such chemical structure modification or synthesis has been performed over decades by what might be termed more classical means. Indeed, the complete synthesis of natural products has been an area of interest for a long time, and the efforts to produce man-made natural products has provided significant challenge and learning opportunity over the years[56,57,63-69]. Because of the widespread chemical diversity that can be found in natural products, an ever-expanding collection of fascinating natural product compounds will continue to be presented to chemists for synthesis[70]. If one compares the chemical diversity of man-made synthetic products with the chemical diversity of natural product compounds, it quickly becomes apparent that there are significant qualitative differences between synthetics and natural products[71]. Natural product compounds contain more alcoholic and ether groups, while pure synthetic compounds possess more aromatics, amines, and amides. If one looks at group combinations, there are higher percentages of alcohol/ether, alcohol/ester, arene/alcohol, arene, alcohol, or ether functionalities in natural product compounds when compared to the synthetic compounds. However, pure man-made synthetic compounds are found to have combinations such as arene/amine, amine/amide, or amine/arene/amide in higher frequencies than natural product type of compounds. Finally, natural product compounds are found to more commonly possess bridgehead atoms and contain a greater number of rotatable bonds per molecule, chiral centers per molecule, and rings per molecule than pure man-made synthetic compounds. The importance of these differences is that they reveal and emphasize the complementarity of natural product compounds as a group with man-made synthetic compounds. Despite all of the knowledge and achievements that have been gained and the advances that have been made, classical synthetic organic chemistry will not alone unlock and open the potential of natural products to the pharmaceutical marketplace. Instead, the future lies in the synergistic union of classical organic chemistry with microbiology, biochemistry, combinatorial chemistry, and other fields to provide new synthetic strategies to generate natural-product-based drugs. The history and specific techniques of combinatorial chemistry are beyond the scope of this review, and the reader is referred to appropriate textbooks for discussions on that topic. While it should be

recognized that combinatorial chemistry is a perfect match for high-throughput screening because of its ability to produce large numbers of compounds in a short period of time. The promise of combinatorial chemistry to deliver more drug candidates within a shorter period of time has remained unfulfilled. What has been lacking in combinatorial chemistry is the skeletal structural novelty that natural products can provide[17,72-82]. Over time, organic synthetic chemists have become interested in enzymes and their potential role in natural product synthesis[83-85]. Enzymes have great power as catalysts for regiospecific and stereocontrolled synthesis. These biological catalysts are very capable at room temperature of converting inexpensive substrates into value-added products at a significantly high throughput. However, barriers remain to the more expanded use of enzymes in organic synthetic chemistry. The most important of such obstacles includes the inability of enzymes to work with unnatural substrates. Combinatorial biosynthesis utilizes enzymes from various natural product source biosynthetic pathways to create novel chemical structures[86-88]. The engineering of polyketide synthases has thus far been the central point of this activity and led to the production of several erythromycin analogs. The end result of such research activity will be the development of more rational and faster methods of production of new compounds for the development of therapeutic agents from natural products. The research on and screening of natural products today is focusing on many different therapeutic indications. Fermentation broths and plant extracts have done well in delivering leads and genomics and molecular biology have done well in delivering targets. Regardless of the type of compounds involved, improved efficiencies in the design and synthesis of natural-product-based agonists and antagonists will be key to a realization of the full potential of natural products as drugs[17,89,90].

Conclusions

Natural product research has been the single most successful strategy for discovering new pharmaceuticals and has contributed dramatically to extending human life and improving clinical practice. As long as Nature continues to yield novel, diverse chemical entities possessing selective biological activities, natural products will play an important role as leads for new pharmaceuticals. An interesting recent example is the alkaloid galantamine (Nivalin, Reminyl), originally isolated from the bulbs of the Amaryllidaceae family (snow-drops, daffodils, etc.), which has found use in the symptomatic treatment of Alzheimer's Disease[91]. It is a reversible and competitive inhibitor of acetylcholinesterase that also interacts allosterically with nicotinic acetylcholine receptors to potentiate the action of agonists. By acting to enhance the reduced central cholinergic function associated with this disease, significant improvements in cognition and behavioral symptoms have been observed in patients. In this case it is the alkaloid itself that is used as the active compound and it will be interesting to see whether development leads to better drugs.

Over 90% of bacterial, fungal, and plant species are still waiting to be investigated[92]. High throughput screening methods will allow even greater numbers of samples to be tested against more biological targets[93,94], although this approach sometimes produces more data than can be conveniently integrated into a research program. An alternative view is that the

elucidation of the biological effects of chosen compounds, in some detail, will yield insight into biological processes that may open avenues for medicinal chemistry research that is not based on pure chance. This view is based on the recognition that secondary metabolites have been produced and ruthlessly selected, by evolution, over a long period of time. Either way, the medicinal chemist has a wonderful opportunity to continue utilizing the rich chemical diversity offered by nature, as is shown in two recent reviews that explore this topic in some detail[95,96].

The best approach for the identification of natural product leads is a matter of debate. Some very inventive techniques have been used in the bioassay-guided method; for example, by spraying TLC plates with reactive media that respond by producing a color change in the presence of an active compound. An alternative is to use an ethnobotanical or ethnopharmacological technique, whereby the accumulated wisdom of many generations of native plant users may be harnessed in the search for better medicines for all. These two techniques may be combined, so that the native people describe the uses to which they put the plant and the researchers devise a bioassay that is used to find the active components. The problem with any bioassay-guided technique, however, is that the inactive constituents are not identified. This represents a considerable waste, given that the plant has had to be collected, preserved, and identified. An alternative view is that it is best to extract all the constituents, with a view to screening in whichever way is appropriate, at that time or in the future. With modern high-performance liquid chromatography facilities it is possible to reduce a plant to its secondary metabolites, as single compounds, in a few days: the products are then able to be screened in a high throughput manner in an equally short time and the compounds can be reevaluated when new screens become available. One thing is certain: the variety of natural product structures, after perhaps 300 million years of natural selection, far exceeds the bounds of human imagination, unlike the typical output from combinatorial chemistry!

Acknowledgement

The presented work is a part of the project No. 172061, supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia.

References

1. Cragg, G. M., Newman, D. J., Snader, K. M. (1997). Natural products in drug discovery and development. *J. Nat. Prod.*, *60*(1), 52–60.
2. Gerardy, R. and Zenk, M. H. (1992). Formation of salutaridine from (*R*)-reticuline by a membrane-bound cytochrome P-450 enzyme from *Papaver somniferum. Phytochemistry*, *32*(1), 79–86.
3. Stone, M. J., Williams, D. H. (1992). On the evolution of functional secondary metabolites (natural products). *Mol. Microbiol.*, *6*(1), 29–34.
4. Newman, D. J., Cragg, G. M., Kingston, D. G. I. (2003). Natural products as pharmaceuticals and sources or lead structures. In C. G. Wermuth (Ed.) Practice of Medicinal Chemistry. Academic Press, Amsterdam, Boston, pp. 91–109.

5. Henkel, T., Brunne, R. M., Muller, H., Reichel, F. (1999). Statistical investigation into the structural complementarity of natural products and synthetic compounds. *Angew. Chem. Int. Ed. Engl.*, *38*(5), 643–647.
6. Feher, M., Schmidt, J. M. (2003). Property distributions: differences between drugs, natural products, and molecules from combinatorial chemistry. *J. Chem. Inf. Comput. Sci.*, *43*(1), 218–227.
7. Lee, M.-L., Schneider, G. (2003). Scaffold architecture and pharmacophoric properties of natural products and trade drugs: application in the design of natural product-based combinatorial libraries. *J. Comb. Chem.*, *3*(3), 284–289.
8. Stahura, F. L., Godden, J. W., Xue, L., Bajorath, J. (2000). Distinguishing between natural products and synthetic molecules by descriptor Shannon entropy analysis and binary QSAR calculations. *J. Chem. Inf. Comput. Sci.*, *40*(5), 1245–1252.
9. Grabowski, K., Schneider, G. (2007). Properties and architecture of drugs and natural products revisited. *Curr. Chem. Biol.*, *1*(1), 115–127.
10. Quinn, R. J., Carroll, A. R., Pham, N. B., Baron, P., Palframan, M. E., Suraweera, L., Pierens, G. K., Muresan, S. (2008). Developing a drug-like natural product library. *J. Nat. Prod.*, *7*(3), 464–468.
11. Ertl, P., Roggo, S., Schuffenhauer, A. (2008). Natural product-likeness score and its application for prioritization of compound libraries. *J. Chem. Inf. Model.*, *48*(1), 68–74.
12. Bugni, T. S., Richards, B., Bhoite, L., Cimbora, D., Harper, M. K., Ireland, C. M. (2008). Marine natural product libraries for high-throughput screening and rapid drug discovery. *J. Nat. Prod.*, *71*(6), 1095–1098.
13. Eldridge, G. R., Vervoort, H. C., Lee, C. M., Cremin, P. A., Williams, C. T., Hart, S. M., Goering, M. G., O'Neil-Johnson, M., Zeng, L. (2002). High-throughput method for the production and analysis of large natural product libraries for drug discovery. *Anal. Chem.*, *74*(16), 3963–3971.
14. Wagenaar, M. M. (2008). Pre-fractionated microbial samples: the second generation natural products library at Wyeth. *Molecules*, *13*(6), 1406–1426.
15. Bindseil, K. U., Jakupovic, J., Wolf, D., Lavayre, J., Leboul, J., van der Pyl, D. (2001). Pure compound libraries; a new perspective for natural product based drug discovery. *Drug Discov. Today*, *6*(16), 840–847.
16. Koehn, F. E., Carter, G. T. (2005). The evolving role of natural products in drug discovery. *Nat. Rev. Drug Discov.*, *4*(3), 206–220.
17. Abel, U., Koch, C., Speitling, M., Hansske, F. G. (2002). Modern methods to produce natural-product libraries. *Curr. Opin. Chem. Biol.*, *6*(4), 453–458.
18. Barrow, R. A. (2005). Isolation of microbial natural products. In S. D. Sarker, Z. Latif, A. I. Gray (Eds.), Methods in Biotechnology. Vol. 20. Natural Products Isolation. Humana, Totowa, NJ, pp. 391–414.
19. Henry, M. C., Yonker, C. R. (2006). Supercritical fluid chromatography, pressurized liquid extraction, and supercritical fluid extraction. *Anal. Chem. 78*(12), 3909–3915.
20. Harrigan, G. G., Goetz, G. H. (2005). Chemical and biological integrity in natural products screening. *Comb. Chem. High Throughput Screen.*, *8*(6), 529–534.
21. Yan, B., Fang, L., Irving, M., Zhang, S., Boldi, A. M., Woolard, F., Johnson, C. R., Kshirsagar, T., Figliozzi, G. M., Krueger, C. A., Collins, N. (2003). Quality control in combinatorial chemistry: determination of the quantity, purity, and quantitative purity of compounds in combinatorial libraries. *J. Comb. Chem.*, *5*(5): 547–559.

22. Cannell, R. J. P. (1998). Follow-up of natural product isolation. *Methods Biotechnol.*, *4* (Natural Products Isolation), 425–463.
23. Cannell, R. J. P. (1998). How to approach the isolation of a natural product. In R. J. P. Cannell (Ed.), Methods in Biotechnology, Vol. 4: Natural Products Isolation. Humana, Totowa, NJ, pp. 1–51.
24. Hill, D. C. (1998). Advanced screening technology and informatics for natural products drug discovery. In A. L. Harvey (Ed.), *Advances in Drug Discovery Techniques.*Wiley, Chichester, pp. 25–37.
25. Demain, A. L. (1998). Microbial natural products: Alive and well in 1998. *Nat. Biotechnol.*, *16*(1), 3–4.
26. Gad, S. C. (2003). Principles of screening and study design. In *Safety Pharmacology in Pharmaceutical Development and Approval.* CRC, Boca Raton, FL, pp. 23–42.
27. Duke, S. O., Dayan, F. E., Romagni, J. G., Rimando, A. M. (2000). Natural products as sources of herbicides: Current status and future trends. *Weed Rese.*, *40*(1), 99–111.
28. Harvey, A. L. (1999). Medicines from nature: Are natural products still relevant to drug discovery? *Trends Pharmacol. Sci.*, *20*(5), 196–198.
29. Woodruff, H. B. (1999). Natural products from microorganisms. An Odyssey revisited. *Actinomycetologica*, *13*(2), 58–67.
30. El Sayed, K. A. (2000). Natural products as antiviral agents. *Stud. Nat. Prod. Chem.*, *24* [Bioactive Natural Products (Part E)], 473–572.
31. Harvey, A. (2001). The continuing value of natural products to drug discovery. *GIT Lab. J.*, *5*(6), 284–285.
32. Grabley, S., Sattler, I. (2003). Natural products for lead identification: Nature is a valuable resource for providing tools. In A. Hillisch and R. Hingenfeld (Eds.), Modern Methods of Drug Discovery. Birkhäuser Verlag, Switzerland, pp. 87–107.
33. Hilton, M. D. (2003). Natural products: Discovery and screening. In V. A. Vinci and S. R. Parekh (Eds.), Handbook of Industrial Cell Cultures. Humana, Totowa, NJ, pp. 107–136.
34. Vlietinck, A. J., Apers, S. (2001). Biological screening methods in the search for pharmacologically active natural products. In C. Tringali (Ed.), Bioactive Compounds from Natural Sources. Taylor & Francis, London, pp. 1–29.
35. Quinn, R. J. (1999). High-throughput screening in natural product drug discovery in Australia utilising Australia's biodiversity. *Drug Devel. Res.*, *46*(3/4), 250–254.
36. Quinn, R. J. (1999). QPRI's system for screening of natural products. *Proc. Phytochem. Soc. Eur.*, *43* (Bioassay Methods in Natural Product Research and Drug Development), 151–157.
37. Mehta, R. G., Pezzuto, J. M. (2002). Discovery of cancer preventive agents from natural products: from plants to prevention. *Curr. Oncol. Rep.*, *4*(6), 478–486.
38. Barrett,D. (2002). From natural products to clinically useful antifungals. *Biochim. Biophys. Acta*, 1587, 224–233.
39. Yang, S. S., Cragg,G. M., Newman,D. J., Bader, J. P. (2001). Natural product-based anti-HIV drug discovery and development facilitated by the NCI Development Therapeutics Program. *J. Nat. Prod.*, *64*(2), 265–277.
40. Claeson, P., Bohlin, L. (1997). Some aspects of bioassay methods in natural product research aimed at drug lead discovery. *Trends Biotechnol.*, 15/17, 245–248.
41. Silva, C. J., Brian, P., Peterson, T. (2001). Screening of combinatorial biology libraries for natural products discovery. *Drugs Pharmaceut. Sci.*, 114 (Handbook of Drug Screening), 357–382.

42. Shimizu, Y. (1998). Purification of water-soluble natural products. *Methods Biotechnol.*, *4* (Natural Products Isolation), 329–341.
43. Wright, A. (1998). Isolation of marine natural products. *Methods Biotechnol.*, *4* (Natural Products Isolation), 365–408.
44. Keim,C., Ladisch, M. R. (2000). Bioseparation of natural products. *Prog. Biotechnol.*, *16* (Bioseparation Engineering), 15–20.
45. Alvi, K. A. (2000). A strategy for rapid identification of novel therapeutic leads from natural products. In S. Cutler and H. Cutler (Eds.), *Biologically Active Natural Products*. CRC, Boca Raton, FL, pp. 185–195.
46. Foucault, A. P., Chevolot, L. (1998). Counter-current chromatography: Instrumentation, solvent selection and some recent applications to natural product purification. *J. Chromatogr.A*, *808*(1–2), 3–22.
47. Foucault, A. P. (1997). Recent advances in purification of natural products by countercurrent chromatography. In P. Schreier (Ed.), *Natural Products Analysis: Chromatography, Spectroscopy, Biological Testing*, Symposium, Wuerzburg, Germany, September 1997. Vieweg, Wiesbaden, Germany, pp. 13–25.
48. Hostettmann, K., Hamburger, M., Hostettmann, M., Marston, A. (1991). New developments in the separation of natural products. *Recent Adv. Phytochem.*, *25* (Modern Phytochemical Methods), 1–32.
49. Hostettmann, K., Marston, A. (1990). Liquid-liquid partition chromatography in natural product isolation. *Anal. Chim. Acta*, *236*(1), 63–76.
50. Horton, T. (1972). Large-scale gel filtration for purification of natural products. *Am. Lab.*, *4*(5), 83–84, 86–88, 90–91.
51. Ellis, N. (2001). Extracting APIs from natural products. *Speciality Chem. Mag.*, *21*(6), 10–12.
52. Kaufmann, B., Christen, P. (2002). Recent extraction techniques for natural products: Microwave-assisted extraction and pressurized solvent extraction. *Phytochem. Anal.*, *13*(2), 105–113.
53. Lang, F., Keller, K., Ihrig, M., Oudtshoorn-Eckard, J., Moller, H., Srinivasan, S., Yu, H. (2001). Biopharmaceutical characterisation of herbal medicinal products. *Pharmazeutische Industrie*, *63*(10), 1005–1010.
54. Puri, R. K. (1998). Supercritical fluid extraction and its applications in natural products. *Ind. J. Nat. Prod.*, *14*(2), 3–23.
55. Mabry,T. J. (2001). Selected topics from forty years of natural products research: Betalains to flavonoids, antiviral proteins, and neurotoxic nonprotein amino acids. *J. Nat. Products*, *64*(12), 1596–1604.
56. Nakanishi, K. (1999). An historical perspective of natural products chemistry. *Comprehensive Nat. Prod. Chem.*, *8*, xxi–xxxviii.
57. Nakanishi, K. (1999). An historical perspective of natural products chemistry. In S. Ushio (Ed.), *Comprehensive Natural Products Chemistry*, Vol. 1. Elsevier Science B.V., Amsterdam, pp. 23–40.
58. Yu, D., Chen, Y., Liang, X. (2001). Structural chemistry and biological activities of natural products from Chinese herbal medicines. *Emerging Drugs*, *1* (Molecular Aspects of Asian Medicines), 215–321.
59. Steinbeck, C. (2001). The automation of natural product structure elucidation. *Curr. Opin. Drug Discov. Devel.*, *4*(3), 338–342.
60. Lindel, T., Junker, J., Köck, M. (1997). COCON: From NMR correlation data to molecular constitutions. *J. Mol. Model*, 3, 364–368.

61. Steinbeck, C. (2001).A platform-independent, distributed, and parallel system for computer-assisted structure elucidation in organic chemistry. *J. Chem. Inf. Comput. Sci.*, 41, 1500–1507.
62. Hemmer, M. C., Gasteiger, J. (2000). Prediction of three-dimensional molecular structures using information from infrared spectra. *Anal. Chim. Acta*, 420, 145–154.
63. Compostella, F., Lay, L. (2001). Critical surveys covering the year 2000:Total syntheses of natural products. In A. Corbella (Ed.), *Seminars in Organic Synthesis, Summer School, 26th*, Gargano, Italy, June 18–22, 2001. Societa Chimica Italiana, Rome, Italy, pp. 453–581.
64. Fallis, A. G. (1999). 1998 Alfred Bader Award Lecture. Tangents and targets: The synthetic highway from natural products to medicine. *Can. J. Chem.*, *77*(2), 159–177.
65. Furstner, A. (1999). Venturing into catalysis based natural product synthesis. *Synlett*, 10, 1523–1533.
66. Marko, I. (2001). Natural product synthesis: The art of total synthesis. *Science*, *294*(5548), 1842–1843.
67. Patchett, A. (2002). 2002 Alfred Burger Award Address in Medicinal Chemistry. Natural products and design: Interrelated approaches in drug discovery. *J. Med. Chem.*, *45*(26), 5609–5616.

68. Righi, P. (1999). Critical surveys covering the year 1998:Total syntheses of natural products. In A. Corbella (Ed.), *Seminars in Organic Synthesis, Summer School, 24th*, Gargano, Italy, June 14–18, 1999. Societa Chimica Italiana, Rome, Italy, pp. 401–518.
69. Sorensen, E. J. (1999). Selected, recent developments in the chemical synthesis of biologically-active natural products. *Curr. Opin. Drug Discov. Devel.*, *2*(6), 606–630.
70. Whitehead, R. (1999). Natural product chemistry. *Annu. Repts. Progr. Chem., Sect. B: Organic Chem.*, 95, 183–205.
71. Müller, H., Brackhagen, O., Brunne, R., Henkel, T., Reichel, F. (2000). Natural products in drug discovery. *Ernst Schering Res. Found.Workshop*, 32, 205–216.
72. Breinbauer, R.,Vetter, I. R.,Waldmann, H. (2002). From protein domains to drugm candidates—Natural products as guiding principles in the design and synthesis of compound libraries. *Ang. Chem. Int. Ed.*, *41*(16), 2878–2890.
73. Breinbeuer, R., Manger, M., Scheck, M., Waldmann, H. (2002). Natural product guided compound library development. *Curr. Med. Chem.*, 9, 2129–2145.
74. Ganesan, A. (2001). Integrating natural product synthesis and combinatorial chemistry. *Pure Appl. Chem.*, *73*(7), 1033–1039.
75. Hall, D. G., Manku, S.,Wang, F. (2000). Solution- and solid-phase strategies for the design, synthesis, and screening of libraries based on natural product templates: A comprehensive survey. *J. Combinatorial Chem.*, *3*(2), 125–150.
76. Mehta, G., Singh, V. (2002). Hybrid systems through natural product leads: an approach towards new molecular entities. *Chem. Soc. Rev.*, *31*(6), 324–334.
77. Nicolaou, K. C., Pfefferkorn, J. A. (2001). Solid phase synthesis of complex natural products and libraries thereof. *Biopolymers*, *60*(3), 171–193.
78. Nielsen, J. (2002). Combinatorial synthesis of natural products. *Curr.Opin. Chem. Biol.*, *6*(3), 297–305.
79. Tsoi, C. J., Khosla, C. (1995). Combinatorial biosynthesis of “unnatural” natural products: The polyketide example. *Chem. Biol.*, *2*(6), 355–362.
80. Von Dohren, H. (1995). Peptides. *Biotechnology*, 28, 129–171.
81. Wessjohann, L. A. (2000). Synthesis of natural-product-based compound libraries. *Curr. Opin. Chem. Biol.*, *4*(3), 303–309.

82. Wilson, L. J. (2000). Recent advances in solid-phase synthesis of natural products, In K. Burgess (Ed.), *Solid-Phase Organic Synthesis*, J Wiley, New York, pp. 247–267.
83. Khosla, C. (2000). Natural product biosynthesis: A new interface between enzymology and medicine. *J. Org. Chem.*, *65*(24), 8127–8133.
84. McCoy, M. (1999). BIOCATALYSIS GROWS FOR DRUG SYNTHESIS. *Chem. Eng. News*, January 4, pp. 10–13.
85. Roessner, C. A., Scott, A. I. (1996). Genetically engineered synthesis of natural products: From alkaloids to corrins. *Annu. Rev. Microbiol.*, 50, 467–490.
86. Borman, S. (1998). Biosynthesis combinatorially. *Chem. Eng.News*, September 14, pp. 29–30.
87. Cordell, G. A. (2002). Recent developments in the study of biologically active natural products. *ACGC Chem. Commun.*, 14, 31–63.
88. Rodriguez, E., McDaniel, R. (2001). Combinatorial biosynthesis of antimicrobials and other natural products. *Curr. Opin. Microbiol.*, *4*(5), 526–534.
89. Rouhi, A. M. (1996). Natural products' scope expanding. IUPAC symposium showcases breadth of approaches chemists use to understand complex molecules from natural sources. *Chem. Eng. News*, *74*(43), 34–43.
90. Rouhi, A. M. (1997). Seeking drugs in natural products. *Chem. Eng.News*, *75*(14), 14–20.
91. Burkhart, C. N. (2000). Ivermectin: an assessment of its pharmacology, microbiology and safety. *Vet. Hum. Toxicol.*, *42*(1), 30–35.
92. Coombes, J. D. (Ed.), (1992) *New Drugs from Natural Sources*, IBC Technical Services, London, pp. 59–62, 93–100.
93. Yarbrough, G. G. , Taylor, D. P., Rowlands, R. T., Crawford, M. S., Lasure, L. (1993). *J. Antibiot.*, *46*(4), 535–544.
94. Moos, W. H., Green, G. D., Pavia, M. R. (1993). Recent Advances in the Generation of Molecular Diversity. In J. A. Bristol (Ed.), Annual Report of Medicinal Chemistry, Vol. 28, Academic Press, New York, pp. 315–324.
95. Shu, Y.-Z. (1998). Recent natural products based development: A pharmaceutical industry perspective. *J. Nat. Prod.*, *61*(8), 1053–1071.
96. Newman, D. J., Cragg, G. M., Snader, K. M. (2000). The influence of natural products upon drug discovery. *Nat. Prod. Rep.*, 17(3), 215–234.

Regulation of Apoptosis in Various Experimental Models of Diseases

Marina Mitrović*

* *Institute of Biomedical Research, Department of Biochemistry, Faculty of Medical Science, University of Kragujevac, Kragujevac , Serbia*
e-mail: mitrovicmarina34@gmail.com

Apoptosis is an important physiological mechanism of cell death used by multicellular organisms to eliminate damaged, infected, unnecessary or potentially dangerous cells, as well as it plays a key role in the pathogenesis of human diseases such as cancer, autoimmune, cardiovascular and neurological disorders. Therefore, therapies that are able to either augmented or limit the initiation of apoptosis are enormous potential to cure many of these diseases. During the past several years of the research on the regulation of apoptosis, rapid progress has been made to identify the molecules that are responsible for the regulation and execution of apoptosis using various experimental models of the diseases including variety of different cancer cell lines, myoma cells lines, as well as knock/out mice treated with the substances that cause various diseases, such as hepatitis. Specifically, this research work revealed the important role of a various proapoptotic and antitapoptotic molecules, such as BAP31, SPIKE, BCL-2, BAX, cytochrome C, caspases, IL33/ST2- receptors in regulation of apoptosis in specific experimental disease models.

Introduction

Apoptosis, a programmed cell death, is an important physiological mechanism of cell death in animal development and tissue homeostasis by which multicellular organisms can eliminate damaged, infected, unnecessary or potentially dangerous cells[1]. In addition, apoptosis plays a fundamental role in the pathogenesis of human diseases. Inhibition or inappropriate activation of apoptosis has been implicated in many forms of human diseases including cancer and autoimmune disorders[2,3]. Conversely, augmented apoptosis contributes to, among others, Alzheimer's and Parkinson's diseases[3]. Therefore, therapies that are able to either augmented or limit the initiation of apoptosis are enormous potential to cure many of these diseases[4].

Apoptosis is characterized by a number of structural and morphological changes that are consequences of the various cell disassembly processes. These changes culminate in the coordinated packaging of cellular contents into fragmented, membrane enclosed cell particles, apoptotic bodies, subsequently eliminated by neighboring epithelial cells via phagocytosis. During early stages of apoptosis, cells undergo morphological changes, such as cell rounding and detachment, plasma membrane blebbing and fragmenation, loss of phosphatidyl serine asymmetry in the cell membrane, and intranucleosomal DNA fragmentation[5].

Apoptotic pathways: extrinsic death-receptor and the intrinsic stress pathways

There are multiple extrinsic and intrinsic initiating death-stimulating pathways that can converge on mitochondria, causing permeabilization of the mitochondrial outer membrane and release of pro-apoptotic proteins that critically contribute to execution of cell suicide. These pathways are activated by diverse proapoptotic inducers such as soluble cytokines such as Tumor Necrosis Factor-α (TNF-α- death ligands, such as Fas[6], transformed oncogenes, such as Ras or Myc, extensive DNA damage[7], oncogenic signaling by E1A[8], cell detachment from the extracellular matrix (anoikis), low growth factor conditions, malfunction of protein folding processes, various cytostatic agents and other examples of apoptotic stresses.
The most intensively studied extrinsic apoptotic pathway is initiated by cell surface death-inducing signaling complex (DISC) through stimulation of FAS/CD95 death receptors. CD-95 belongs to the tumour-necrosis factor receptor (TNFR) family and functions in the removal of activated T cells at the end of the immune response. Binding of extracellular ligand, such as Fas, to these receptors induces trimerization of the receptor. This trimerization leads to the recruitment of adapter protein FADD (Fas Associated Death Domain) through the homotypic interactions between death domains (DD) of FADD and cytosolic tail of death receptor. FADD then recruits procaspase-8 to the complex through the interactions between death effector domains (DED) present on both FADD and procaspase-8. These recruitments subsequently lead to the activation of this initiator caspase. In type I cells[9], activation of caspase 8 can initiate a downstream cascade of events by directly processing effector procaspases such as procaspase 3, which further contribute to execution of apoptosis(Figure 1A). In type II cells[9], activated caspase-8 requires the death input by the mitochondrial pathway to amplify the death signal received from the cell surface (Figure 1B). Active caspase 8 cleaves and activates the regulators of the mitochondrial intrinsic apoptotic pathway, the cytosolic target BID, and ER resident protein BAP31[10,11]. Cleavage of BID, a pro-apoptotic BH3-only family member of BCL-2 family, by caspase 8 generates tBID, which translocates to mitochondria and induces the release of intermembrane pro-apoptotic factors, including cytochrome c, Smac/Diablo, IAPs, Omi/HtrA2, AIF and endonuclease G, into the cytosol. Released cytochrome C, together with procaspase 9 and Apaf-1, become integral component of the apoptosome, which like caspase-8 subsequently activates downstream effector procaspases, such as caspase-3, -6, and -7, and apoptosis.

Regulators of apoptosis: a BCL-2 family

BCL-2 family proteins are the major regulators of the apoptosis as well as the executioners of death signals at the mitochondria. They are both membrane localized and soluble proteins, found in numerous locations in the cells, including cytosol, mitochondria[12], ER[13]. Their localization to different organelles is critical for their effect on apoptosis. Members of BCL-2 family include pro- and anti-apoptotic proteins which are classified according to their content of Bcl-2 homology (BH) domains[14]. The family can be divided into three main groups. The anti-apoptotic multidomain proteins, which include BCL-2, BCL-XL, MCL1, A1, and

BCL-W, share homology in all 4 available BH domains. The pro-apoptotic multidomain proteins include BAX and BAK and posses sequence homology in BH1-BH3. However, the proapoptotic BH3-only members, such as BID, BAD, BIK, BIM, HRK, PUMA, NOXA and recently identified SPIKE[15], share sequence homology within α-helical BH3 domain required for their apoptotic action. Both multidomain anti-apoptotic and pro-apoptotic BCL-2 members, but only BIK and BIM BH3-only members, contain a hydrophobic transmembrane (TM) domain at their C-terminus required for their targeting to different organelles.
BCL-2 family members are able to form homo and heterodimers resulting in conformational changes causing either their inhibition or activation. Anti-apoptotic members can heterodimerize with pro-apoptotic members through interaction with their deep groove, formed by helices 1 and 2, and exposed BH3 helix on the surface of pro-apoptotic members, thus inhibiting or activating their proapoptotic action. In response to upstream death signaling, many BH3-only proteins, as well as BAX and BAK, must undergo conformational changes that mediate the exposure of BH3 domain and thus interaction with anti-apoptotic BCL-2 members. Moreover, the individual BH3-only members can bind selectively to different multidomain BCL-2 members. For example, some BH3-only proteins, including BAD and NOXA, preferentially bind and inhibit anti-apoptotic members. However, other BH3-only members, such as BID and BIM, can bind and either inhibit anti-apoptotic members or activate multidomain pro-apoptotic members BAX and BAK. However, expression of all of BH3-only members results in the activation of BAX and BAK, which are essential effector molecules in executing mitochondrial dysfunction. Therefore, the outcome of the death signals regulated by BCL-2 proteins depends on the three way ratio of the anti-apoptotic, multidomain pro-apoptotic and BH3-only members (Figure 1B)[12].

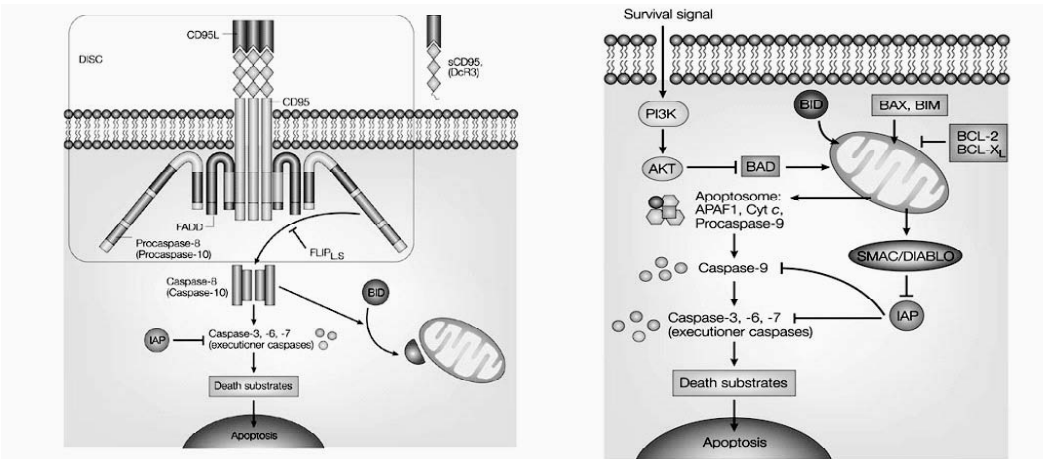


Figure 1. Apoptotic pathways: A) extrinsic death-receptor and B) the intrinsic mitochondrial stress pathways

BAP31 and regulation Apoptosis in cancer cells

In addition to its predicted role in regulating the export of newly synthesized membrane proteins from ER, BAP31 was recognized as regulator of apoptosis in various cancer cell lines soon after its discovery. Previous and current work in our laboratory has identified BAP31 as an interacting partner of antiapoptoic BCL-2 and BCL-XL proteins at the endoplasmic reticulum (ER)[11]. It is both an upstream regulator of the novel procaspase-8 isoform, procaspase-8L, in response to oncogenic death signaling by E1A[16] and a target of caspase 8 whose cleavage sensitizes the mitochondrial apoptotic pathway to BID[17, 18]. The cytosolic domain of BAP31 contains a degenerate Death Effector and overlapping Coiled Coil (DECC) domain flanked on either side by identical caspase-8 recognition sites (AAVD.G) and terminating in a canonical KKXX ER retrieval signal. Following activation of caspase 8, as the most proximal event in the FAS death pathway and presumably following pro-caspase 8L activation at the ER, BAP31 is efficiently cleaved at both caspase 8 sites, generating a membrane integrated p20 fragment that is a potent inducer of apoptosis when expressed ectopically, arguing that cleavage of BAP31 plays a role as a potential regulator of apoptosis . Consistent with this, caspase-resistant mutant BAP31 (crBAP31) strongly resist FAS-induced apoptotic membrane blebbing and fragmentation and release of cytochrome c from mitochondria, despite the fact that caspases are active in these cells.[18] These results indicate a role for BAP31 in regulating cytochrome c release from the organelle in intact cells and suggest the contribution of BAP31 caspase cleavage to cytoplasmic apoptosis during FAS mediated death.

To better understand the contribution of BAP31 in this ER-mitochondrial crosstalk during FAS signaling, we further studied the involvement of uncleaved BAP31 in this pathway. During this research I generated a stable cell line in Bap31 knockout mouse cells that expresses crBAP31, in which the endogenous p20 pro-apoptotic cleavage product cannot be generated following chemical activation of ectopic caspase 8 (FKBP-casp8) by the compound FK1012Z. In this system, uncleaved Bap31 acts as a direct inhibitor of caspase–8 proximal events by conferring resistance to cytochrome c release and cellular condensation[17]. In addition, a new component of the BAP complex was identified, the putative ion channel of the ER, A4, which cooperates with crBAP31 to inhibit FAS-initiated release of cytochrome c from mitochondria. Moreover, we further investigated the role of the p20 caspase cleavage fragment of BAP31 at the ER in modulating mitochondrial dysfunction during apoptosis (Figure 2). The p20 fragment, which remains integrated in the ER membrane, induces mitochondrial fission through ER Ca^{2+} signals, enhancing cytochrome c release to the cytosol in cooperation with other caspase-8 generated stimuli including tBID[18]. Thus, the opposing effect of uncleaved BAP31, as an inhibitor, and cleaved p20BAP31 as an activator of apoptosis puts BAP31 in the group of key anti-apoptotic regulators that are converted from inhibitors to promoters of apoptosis by caspase cleavage[19].
Thus, BAP31 plays important roles in apoptosis both as a potential procaspase-8 activation complex and as a key substrate for this enzyme in mediating ER-driven sensitization of the mitochondrial apoptotic pathway to other “hits” at this organelle.

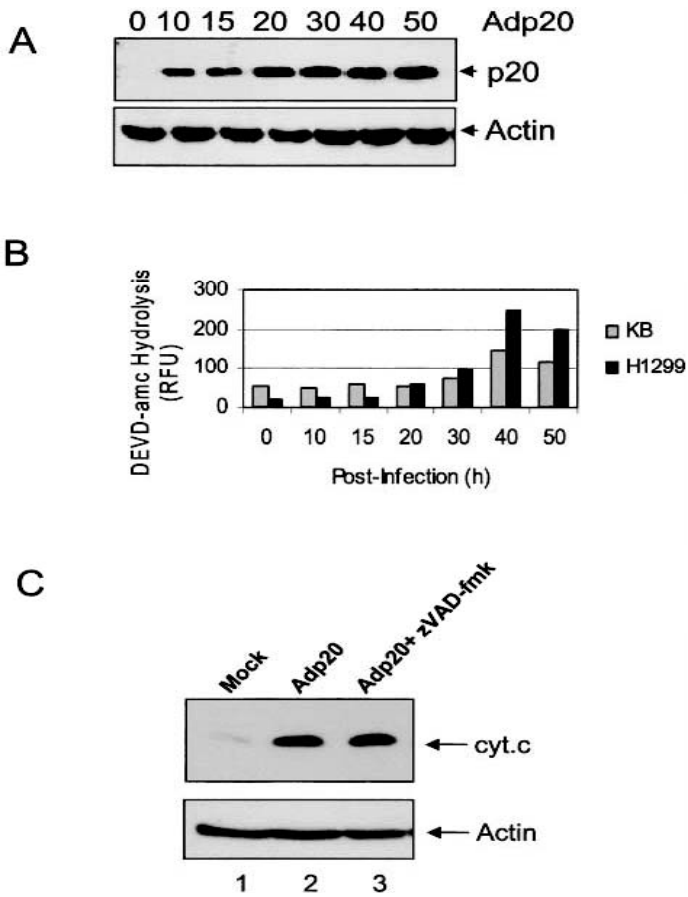


Figure 2. Prolonged expression of BAP31- p20 caspase cleavage product induces mitochondrial apoptosis. (A) Expression of p20 in KB cells. Cells were infected with Adp20 and cell lysates were collected and analyzed by immunoblotting at the times indicated post-infection. (B) KB and H1299 cells were infected with Adp20, and effector caspase (DEVDase) activity was measured at the indicated times post-infection by the ability of cell lysates to hydrolyze the fluorogenic caspase substrate DEVD-amc. Shown is a representative experiment. (C) KB cells were mock infected or infected with Adp20 for 35–40 h in the absence or presence of 50 M zVAD-fmk, and equivalent amounts of post-mitochondrial supernatants were analyzed for the presence of cyt.c by SDS-PAGE and immunoblotting.

BAP31 and its caspase cleavage product in regulating cell surface expression of tetraspanins and integrin-mediated cell survival induced by serum starvation

Although BAP31 role as an important regulator of apoptotic death has been well characterized, BAP31 role in normal cell physiology has recently emerged as a focus for biochemical research. Indeed, BAP31 has been described as a potential chaperone/quality control factor proposed to regulate the transport of certain membrane proteins from the ER to the plasma membrane[20]. Regulation and maintenance of the functional integrity of the plasma membrane is highly important to the normal cell physiology. Numerous diseases and pathological disorders including cystic fibrosis, retinal, cardiac and liver diseases, mental retardation etc., are associated with defects in the regulation of the transport of newly synthesized proteins out of ER to plasma membrane.

BAP31, a resident integral protein of the endoplasmic reticulum (ER) membrane, regulates the export of other integral membrane proteins to the downstream secretory pathway. Here we show that cell surface expression of the tetraspanins CD9 and CD81 is compromised in mouse cells deleted of the *Bap31* gene. CD9 and CD81 facilitate the function of multiprotein complexes at the plasma membrane, including integrins. Of note, BAP31 does not appear to influence the egress of $\alpha5\beta1$ or $\alpha v\beta3$ integrins to the cell surface but in *Bap31*-null mouse cells these integrins are not able to maintain cellular adhesion to the extracellular matrix in the presence of reduced serum (Figure 3). Consequently, *Bap31*-null cells are sensitive to serum starvation-induced apoptosis. Reconstitution of wt BAP31 into these *Bap31*-null cells restores integrin-mediated cell attachment and cell survival after serum stress, whereas interference with the functions of CD9, $\alpha5\beta1$ or $\alpha v\beta3$ by antagonizing antibodies makes BAP31 cells act like *Bap31*-null cells in these respects. Finally, in human KB epithelial cells protected from apoptosis by BCL-2, the caspase-8 cleavage product, p20 BAP31, inhibits egress of tetraspanin and integrin-mediated cell attachment. Thus, p20 BAP31 can operate upstream of BCL-2 in living cells to influence cell surface properties due to its effects on protein egress from the ER[21].

SPIKE, a cytosolic pro-apoptotic molecule induces mitochondrial apoptosis in cancer

Proteins of the BCL-2 family are important regulators of apoptosis. The BCL-2 family includes three main subgroups: the anti-apoptotic group, such as BCL-2, BCL-XL, BCL-W, MCL-1; multi domain pro-apoptotic BAX, BAK: and pro-apoptotic “BH3 only” BIK, PUMA, NOXA, BID, BAD and SPIKE. SPIKE, a rare pro-apoptotic protein, is highly conserved throughout the evolution, including C. Elegans, whose expression is downregulated in certain tumors, including kidney, lung and breast. In the literature, SPIKE was proposed to interact with BAP31 and prevent BCL-XL from binding to BAP31. In this research project we utilized the Position Weight Matrix method *to identify SPIKE* to be a BH3 only pro-apoptotic protein mainly localized in the cytosol of all cancer cell lines tested including Human

293T embryonic kidney, H1299 lung carcinoma, HeLa cervical carcinoma,and KB oral epithelial carcinoma. Overexpression of SPIKE weakly induced apoptosis in comparison to the known BH3-only pro-apoptotic protein BIK. SPIKE promoted mitochondrial cytochrome C release, the activation of caspase 3 and the caspase cleavage of caspase's downstream substrates BAP31 and p130CAS. Although the informatics analysis of SPIKE implicates this protein as a member of the BH3-only BCL-2 sub-family, its role in apoptosis remains to be elucidated[15].

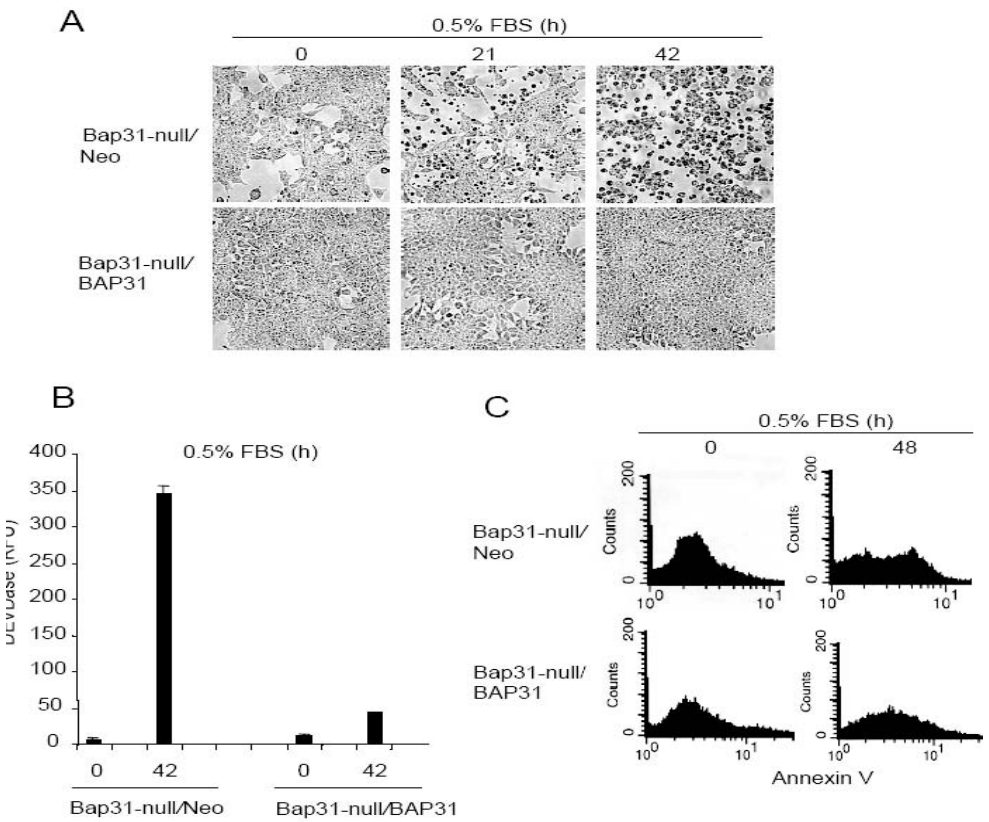


Figure 3. Deletion of Bap31 sensitizes cells to serum starvation-induced cell death. (A) Morphologies of the Bap31-null and Bap31-null/BAP31 cells were examined by phase-contrast microscopy following 21 and 42 hr culture under serum starvation conditions (0.5% FBS). **(B)** Cells were cultured for 0 and 42 h following exposure to reduced serum (0.5% FBS) and DEVDase activity determined by incubating cell lysates (normalized to equivalent protein) with DEVD-amc. Shown are the means ± standard deviations from 3 independent determinations. **(C)** Cells were exposed to 0 or 48 h 0.5% FBS, stained with annexin V, and the fluorescence intensity determined by FACS analysis (Counts = relative fluorescence intensity).

Antiapoptotic - Protective Role of IL-33/ST2 Axis in Con A-Induced Hepatitis

Acute liver failure is associated with significant mortality and could be triggered by autoimmune hepatitis, viral hepatitis, alcohol consumption and hepatotoxins. The underlying pathophysiological mechanisms are still incompletely understood and therefore therapeutic options are limited. Cell death by apoptosis is a prominent feature in a variety of liver diseases[22]. Concanavalin A induced liver injury is an established murine model of T cell mediated hepatitis. We used this model to study the role of Interleukin 33 and its receptor ST2 in the induction of inflammatory pathology and hepatocellular damage. We tested sucseptibility to Concanavalin A induced hepatitis in ST2 deficient and wild type BALB/c mice and analyzed the effects of single injection of Interleukin 33 as evaluated by liver enzyme test, quantitative histology, mononuclear cell infiltration, cytokine production and markers of apoptosis in the liver.

We observed that ST2 deficient mice developed significantly more severe hepatitis as evaluated by biochemical and histological criteria. These findings correlated with significantly higher number of mononuclear cells in the liver, with statistically higher number of CD4+ and CD8+ T cells, NK and NKT cells and in particular F4/80+ macrophages. The level of pro-inflammatory TNF alpha, IFN gamma and IL-17 was also higher in sera of ST2 deficient mice. In contrast, number of CD4+Foxp3+ cells was statistically higher in Wild type mice. Additionally, treatment of Wild type mice with single (1μg) injection of Interleukin 33 led to attenuation of the liver injury and milder infiltration of T cells. Interleukin 33 also inhibits concanavalinA induced apoptosis in liver by suppressing the activation of caspase 3, prevented the expression of BAX and enhanced the expression of antiapoptotic Bcl-2 in the liver (Figure 4). We concluded that Interleukin 33/ST2 axis downregulates Concanavalin A induced liver injury and should be evaluated as potential target in fulminant hepatitis in humans[23].

Myotrexate induce apoptosis via mytochondrial pathway in uterus myoma *in vitro*

Uterine myomas (fibroids) are benign tumors of the myometrium that mostly develop in reproductive-aged women. Local aplication of low doses of Methotrexate (MTX) has been implicated as an effective treatment of the myoma uteri. Myotrexate is a promissing new drug for noninvasive treatment of fibroids. The aim of this study was to investigate cytotoxic and apoptotic effects Myotrexate in T hESC cells. In the study, human fibroblasts derived from uterine myoma (T hESC cell line) were used. The experimental group of cells was treated with Myothrexate at the concentrations of 1, 8 and 16 μM) for 24h, while the control group represented untreated cells. The cytotoxicity was assayed by MTT test and apoptosis was evaluated by Annexin V-FITC assay. The mechanism of the apoptotic effect of Myothrexate was determined by immunofluorescence, a method used to detect both the localization and expression of cytochrome c and caspase 3. Myotrexate inhibited proliferation and induced apoptosis in T hESC cells at all tested doses. Myotrexate treatment of the cells significantly

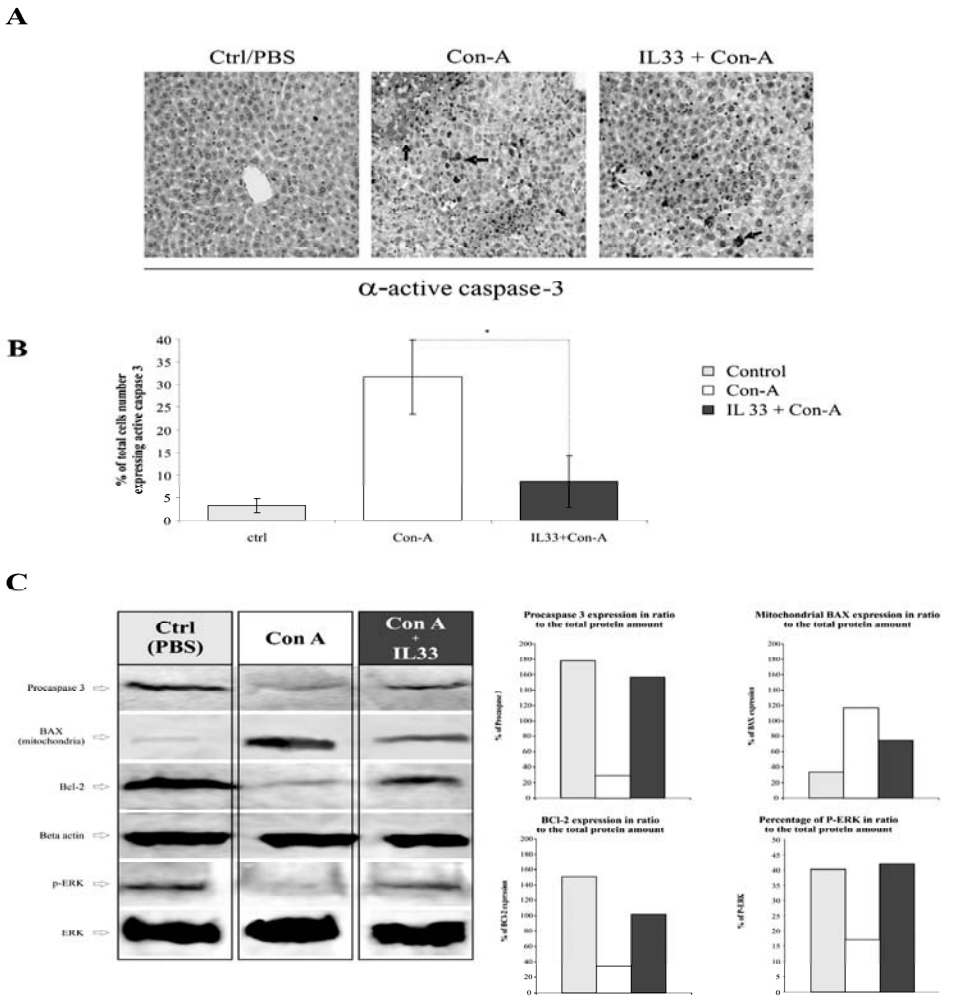


Figure 4. IL-33 suppressed pro-apoptotic and enhanced anti-apoptotic genes
A. Immunohistochemical analysis of active caspase-3 in the paraffin-embedded liver tissue sections after Con A injection.**B**. The percentage of total cells number expressing active caspase-3. The highest expression of activecaspase-3 was observed in the Con-A treated group (open bars) compared both to the control (light greybars) and IL-33 pre-treated mice (dark grey bars). Combined results of 2 experiments with total of 5animals per group (Mean +/-SE; p<*0.05) **C.** Western Blot analysis of protein expression in the liver homogenates. The expression of mitochondrial BAX was attenuated and the expression of procaspase 3, Bcl-2 and p-ERK were increased in the liver homogenates of mice pretreated with rIL-33 (dark grey bars) compared with Con A only treated mice (open bars).

increased the percentage of apoptotic cells in the experimental group compared to the control group. T hES cells stimulated with Myotrexate showed diffuse staining of cytochrome c indicative of its release from mitochondria to the cytosol and subsequent caspase-3 activation when compared to control cells. Myothrexate induces apoptosis in T hES cells and it is most likely that its mechanism of action involves the mitochondrial apoptotic pathway[24].

Acknowledgements

BAP31 studies were supported by the National Cancer Institute of Canada, and the Canadian Institutes of Health Research.IL33/ST2 study is supported by grants: ON175069, ON175071 and ON175103 from Ministry of Science and Technology, Republic of Serbia and grant JP11/10 from Faculty of Medicine University of Kragujevac, Serbia.

References

1. Danial NN, Korsmeyer SJ. (2004). Cell death: critical control points. *Cell.* 116:205-19.
2. Green DR, Evan GI. (2002). A matter of life and death. *Cancer Cell.* Feb;1(1):19-30.
3. Maiese K, *et al.,* (2012) Targeting disease through novel pathways of apoptosis and autophagy. *Expert Opin Ther Targets.* [Epub ahead of print] Aug 27
4. Nicholson DW. (2000). From bench to clinic with apoptosis-based therapeutic agents. *Nature*. Oct 12;407(6805):810-6. Review.
5. Mills JC, Stone NL, Pittman RN. (1999). Extranuclear apoptosis. The role of the cytoplasm in the execution phase. *J Cell Biol.* 146:703-8.
6. Lavrik IN, Krammer PH. (2012) Regulation of CD95/Fas signaling at the DISC. *Cell Death Differ.* Jan;19(1):36-41.
7. Aziz K, *et al.,* (2012). Targeting DNA damage and repair: embracing the pharmacological era for successful cancer therapy. *Pharmacol Ther.*133(3):334-50. Epub 2011 Dec 14.
8. Liao Y, Yu D, Hung MC, (2007). Novel approaches for chemosensitization of breast cancer cells: the E1A story. Adv Exp Med Biol.;608:144-69. Review.
9. Vermeulen, K., Van Bockstaele, D.R., Berneman, Z.N. (2005) Apoptosis: mechanisms and relevance in cancer. *Ann Hematol*. 84:627-39.
10. Li H, Zhu H, Xu CJ, Yuan J. (1998). Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell.* 94:491-501.
11. Ng FW, Shore GC. (1998). Bcl-XL cooperatively associates with the Bap31 complex in the endoplasmic reticulum, dependent on procaspase-8 and Ced-4 adaptor. *J Biol Chem.* 273:3140-3.
12. Estaquier J, *et al.,* (2012). The mitochondrial pathways of apoptosis. *Adv Exp Med Biol*.;942:157-83.
13. Germain M, Mathai JP, Shore GC. (2002). BH-3-only BIK functions at the endoplasmic reticulum to stimulate cytochrome c release from mitochondria. *J Biol Chem.* 277:18053-60.
14. Adams JM, Cory S. (2001). Life-or-death decisions by the Bcl-2 protein family. *Trends Biochem Sci.* 26:61-6.
15. Nikolic I, *et al.,* (2010). Cytosolic pro-apoptotic SPIKE induces mitochondrial apoptosis in cancer. *Biochem Biophys Res Commun.*395(2):225-31. Epub 2010 Mar 31.
16. Breckenridge DG, Shore GC (2000). Regulation of apoptosis by E1A and Myc oncoproteins. *Crit Rev Eukaryot Gene Expr.*;10(3-4):273-80. Review.

17. Wang B, *et al.,*(2003). Uncleaved BAP31 in association with A4 protein at the endoplasmic reticulum is an inhibitor of Fas-initiated release of cytochrome c from mitochondria. *J Biol Chem*. 278(16):14461-8.
18. Breckenridge, D.G., *et al.,* (2003). Caspase cleavage product of BAP31 induces mitochondrial fission through endoplasmic reticulum calcium signals, enhancing cytochrome c release to the cytosol. *J Cell Biol.* 160, 1115-27
19. Cheng EH, *et al.,* (1997). Conversion of Bcl-2 to a Bax-like death effector by caspases. *Science.* 278:1966-8.
20. Wang B, *et al.,* (2008). BAP31 interacts with Sec61 translocons and promotes retrotranslocation of CFTRDeltaF508 via the derlin-1 complex. *Cell*.;133(6):1080-92.
21. Stojanovic, M., *et al.,* (2005). BAP31 and its caspase cleavage product regulate cell surface expression of tetraspanins and integrin- mediated cell survival. *J Biol Chem.* 280, 30018-24.
22. Kahraman A, Gerken G, Canbay A, (2010). Apoptosis in immune-mediated liver diseases. *Dig Dis*.;28(1):144-9. Epub 2010 May 7.
23. Volarevic V, *et al.,* (2012). Protective role of IL-33/ST2 axis in Con A-induced hepatitis. *J Hepatol*. 56(1):26-33.
24. Kastratovic T, Mitrovic M, Nikolic I, Arsenijevic S. Methotrexate and Myotrexate induce apoptosis in human myoma fibroblasts (T hES cell line) via mytochondrial pathway. *Toxicology in Vitro. Submitted* Ms. Ref. No.: TIV-D-12-00075

CXCL12 and PARP-1 are potential key molecules in the promotion of β-cell survival and diabetes attenuation

Nevena Grdović*

* *Department of Molecular Biology, Institute for Biological Research "Siniša Stanković", University of Belgrade, Serbia*
e-mail: nevenag@ibiss.bg.ac.rs

Diabetes mellitus is chronic metabolic disease that results from pancreatic β-cell death or dysfunction. In both type 1 diabetes (T1D), and type 2 diabetes (T2D) the β-cell mass is reduced and the remaining β-cells are stressed by the glucotoxic effects of sustained hyperglycemia and subsequent oxidative stress. Current strategies in diabetes management are directed at lowering blood glucose levels and treating the pathological consequences of diabetes, rather than its causes. Since a common feature of diabetes is a reduction in β-cell mass, developing novel therapeutic treatments and related therapeutics that control and promote β-cell growth and survival are highly desirable. The chemokine CXCL12 and its receptor CXCR4 plays a crucial role in numerous biological processes since they are required for proper cell localization throughout the body and are involved in many aspects of cell survival, tissue repair and regeneration. CXCL12 is emerging as a potential agent for treating diabetes based on its capability to promote β-cell survival through activation of the antiapoptotic and prosurvival Akt kinase. However, it has been shown that β-cell loss that underlies diabetes development is at least in part mediated by necrotic cell death. Programmed necrosis is frequently mediated by overactivation of PARP-1, an abundant nuclear enzyme that is activated in response to various cellular stresses. PARP-1 inhibition or ablation results in improved β-cell viability and prevention of diabetes development in animal models. Here we suggest that CXCL12 exerts its prosurvival effects on β-cells by modulating PARP-1 activity. Crosstalk between CXCL12 and PARP-1 would enable a wider range of prosurvival mechanisms which would not be limited only on preventing apoptotic cell death, but on preventing cell death through necrosis. These new discoveries support the use of CXCL12 as a potential therapeutic for diabetes treatment.

Introduction

Diabetes mellitus is one of the most prevalent diseases in both the developed western world and under-developed countries[1]. According to the World Health Organization, some 346 million people worldwide suffer from diabetes and this number is constantly increasing. Diabetes is a chronic disease, a metabolic disorder characterized by hyperglycemia

which results from disrupted signaling of the hormone insulin, either because of insufficient insulin levels or an unresponsiveness of target cells to insulin action. The diabetic state and in particular uncontrolled hyperglycemia, promotes the development of tissue and organ dysfunction leading to long-term damage of the cardiovascular system, of the nerves in many different parts of the body, as well as of the eyes, kidneys and liver. Although long-term complications of diabetes develop gradually, they are often disabling or even life-threatening. Indeed, the World Health Organization has estimated that 3.4 million people have died from diabetes and its complications in 2004 and that diabetes-related deaths will double between 2005 and 2030. Therefore, finding ways to treat and cure this disease has become a priority.

As the final targets for clinical intervention, different diabetic complications attract more attention then the insulin-secreting pancreatic β-cells which assume a central place in the onset and development of diabetes. Although different etiologies underlie the two major forms of diabetes, diabetes types 1 (T1D) and 2 (T2D), β-cell death and/or their dysfunction are at the core of diabetic pathophysiology[2].

T1D or insulin-dependent diabetes mellitus is caused by the autoimmune destruction of insulin-producing β-cells residing in the pancreatic islets of Langerhans[3]. The infiltration of inflammatory cells into the islets of Langerhans, termed insulitis, results in the selective destruction of β-cells[4]. Insulitis is a common pathological feature of T1D and at the clinical onset of the disease, β-cells are virtually absent. The loss of insulin-producing β-cells results in insulin deficiency and hyperglycemia, with patients requiring daily treatment with insulin to sustain their lives.

Although the primary cause(s) of TD2 is unknown, insulin resistance plays an early role in its pathogenesis. Insulin is central to regulating glucose uptake in insulin-sensitive tissues such as muscle, adipose tissue and liver. Insulin-resistance reduces glucose uptake which in turn results in a rise of blood glucose. In time the hyperglycemia develops into a chronic state, and the sugar that serves as an energy fuel under physiological conditions becomes toxic as it induces adverse effects on cellular functions and structures in different tissues, a phenomenon referred to as glucotoxicity[5]. In addition, hyperglycaemia is commonly associated with deregulation of lipid metabolism and increased levels of free fatty acids (FFA) which also contributes to further β-cell dysfunction (lipotoxicity)[6]. The toxic metabolic environment generated by hyperglucaemia together with elevated FFA is termed glucolipotoxicity[7]. Regardless of the primary cause, chronic hyperglycemia and glucolipotoxicity are accompanied by progressive disruption of normal β-cell functioning, insulin secretion and β-cell loss.

Increased ROS levels induce β-cell dysfunction

Glucotoxicity is mediated at least in part by excess generation of reactive oxygen species (ROS) and other free radicals[8]. Based on elevated oxidants and markers of oxidative tis-

sue damage, it has been documented that patients with diabetes are exposed to chronic oxidative stress[9]. ROS can be generated in β-cells, as in other cells, by several biochemical pathways including glucose autoxidation[10], mitochondrial oxidative phosphorylation[11], activation of the NADPH oxidases[12], nonenzymatic glycation of proteins and consequent activation of advanced glycation end product (AGE) receptors[13] and overloading of the hexosamine and polyol pathways[14]. Chronic exposure of β-cells to high glucose, therefore leads to excess ROS production which disturbs insulin secretion and lowers β-cell viability through multiple mechanisms.

One of the important effects of oxidative stress in β-cells is to disrupt the mechanisms of normal cellular functions. Oxidative stress decreases the expression and activity of pancreatic and duodenal homeobox 1 (PDX-1) and V-maf musculoaponeurotic fibrosarcoma oncogene homolog A (MafA), transcription factors which play central roles in the regulation of multiple genes involved in β-cell function, including the proinsulin gene[15,16]. Prevention of the loss of insulin gene expression by antioxidant enzyme overexpression supports the involvement of oxidative stress in glucose-mediated toxicity[9]. PDX-1 was also shown to regulate β-cell susceptibility to the cellular stress response related to the endoplasmic reticulum or ER stress[17], and to possess antiapoptotic and proliferative activities that help maintain β-cell mass[18]. In addition, elevated ROS may lead to activation of mitogen-activated protein (MAP) kinases and transcription factors, such as nuclear factor κB (NF-κB) and activator protein 1 (AP-1), thus inducing the expression of proapoptotic genes encoding for the BH3 interacting-domain death agonist (Bid) and Bcl-2-associated death promoter (Bad), and downregulating the antiapoptotic gene encoding for B-cell lymphoma-extra large (Bcl-xL) protein[19,20]. These events increase the susceptibility of β-cells to apoptosis under conditions of oxidative stress typically observed in hyperglycaemia. In this way, gluco- and lipotoxicity and significantly raised ROS levels are important contributing factors to β-cell dysfunction, irregular insulin secretion and β-cell loss, and hence diabetes progression.

To prevent ROS-induced cellular structural damage and changes of molecular mechanisms, organisms have developed a defensive mechanism which is supported by the antioxidative system which includes three main groups of antioxidants. The primary antioxidants that constitute the first line of defense include the antioxidant enzymes that work by preventing the formation of new free-radical species. The secondary antioxidants trap radicals, thereby preventing chain reactions, and include the vitamins E and C, β-carotene, uric acid, bilirubin, and albumin. The tertiary antioxidants repair molecules damaged by free radicals, notably DNA, the damage of which is one of the most deleterious effects of free radicals.

Superoxide anion, the parental form of intracellular ROS, can be converted to hydrogen peroxide by enzyme superoxide dismutase (SOD), and then to oxygen and water by several antioxidant enzymes like catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR). However, β-cells are among the least well equipped in terms of intrinsic antioxidant enzyme expression. Thus, their superoxide anion-inactivating SODs in the cytosol

and mitochondria are at levels of about 50% of those observed in the liver[21]. However, the amounts of hydrogen peroxide-inactivating enzymes GPx and CAT are extremely low in β-cells, at levels of only about 1% of those in the liver[21,22]. This apparent imbalance favors hydrogen peroxide accumulation in response to increased ROS production. Recently it was hypothesized that the hydrogen peroxide derived from glucose metabolism, serves as one of the metabolic signals for glucose-stimulated insulin secretion (GSIS)[23]. Therefore, according to the current hypothesis, this vulnerability to reactive species is the price β-cells have to pay in order to fulfill their insulin secreting function[24].

However, ROS are required for proper β-cell functioning

GSIS is regulated by the rate of glucose metabolism in β-cells. The model that explains the triggering of insulin secretion includes the following sequence of events: glucose entry into the β-cell, oxidative glucose metabolism in the cytosol and mitochondria, a rise in the ATP/ADP ratio, closure of K_{atp} chanels and subsequent membrane depolarization, opening of voltage-dependent Ca^{2+} channels, a rise in cytosolic Ca^{2+} and exocytosis and insulin secretion[25,26]. However, the list of signals that couple glucose metabolism to insulin secretion is still incomplete. A growing body of evidence supports the idea that hydrogen peroxide derived from glucose metabolism serves as an intracellular messenger for insulin secretion[27]. ROS generation and thereby hydrogen peroxide generation, occurs in glucose metabolism and is correlated with insulin secretion. It has been shown that exogenously added hydrogen peroxide causes a rapid elevation in insulin release in rat and mouse islets and INS-1 cells under basal conditions, whereas treatment with antioxidants inhibits GSIS[28,29,30]. Hydrogen peroxide-stimulated insulin secretion is a Ca^{2+}-dependent process, suggesting that hydrogen peroxide is critically involved in the influx of $Ca^{2+}$from the extracellular space to the cytosol which allows exocytosis of insulin vesicles[30]. The involvement of reactive species such as hydrogen peroxide in GSIS places pancreatic β-cells in an extraordinary situation. For their function as glucose sensors and producers of insulin, β-cells requires an intracellular milieu rich in oxygen and glucose in order to generate the signal for increased insulin secretion in response to elevated glucose concentration. However, chronically raised glucose levels intensify metabolic reactions and resulting ROS overproduction. This paradoxical situation is reason for caution in basing a strategy against β-cell loss and/or dysfunction on the indiscriminate increase of activity of the antioxidant system of β-cells.

As β-cells have an especially limited capacity for inactivation of reactive species, their only chance of survival and proper functioning is by maintaining a redox balance. Hence the idea that an increased antioxidant status could improve β-cell tolerance to oxidative stress has been extensively examined. Overexpression of SOD, CAT, either alone or together, was shown to provide protection against oxidative stress-induced β-cell death and diabetes in various model systems[31,32,33,34]. Also, treatment with antioxidants such as N-acetyl-cysteine (NAC) or aminoguanine (AG) preserve the insulin content and insulin

gene expression[9]. However, in spite of this protective role, antioxidant molecules and antioxidant enzymes can suppress the ROS-mediated signaling pathway and thereby decreasing GSIS[24,35]. For this reason, attempts at improving the antioxidant system in β-cells has proved to be "too much of a good thing". By attenuating glucose-dependent ROS signaling and insulin secretion this can further endanger β-cell functionality. Studies have confirmed a hypothesis according to which overexpression of hydrogen peroxide-scavenging enzymes (CAT and GPx) actually increases the likelihood of developing diabetes[36,37]. In addition, therapeutic approaches performed in large scale trials with selected antioxidants and T2D patients have yielded disappointing results, without any major improvements in long-term outcome[38]. Therefore, the paradoxical role of ROS in β-cell function strongly suggests that other approaches need to be considered for treating diabetes.

Current strategies for treating diabetes

β-cell dysfunction and its consequences are currently treated in several different ways. Insulin replacement therapy is used in T1D and advanced stages of T2D. Insulin therapy is life-saving, however, it does not restore normoglycemia since postprandial levels of glucose continue to be excessively high in individuals on insulin replacement therapy. Thus, insulin therapy must be delivered by multiple daily injections or continuous infusion and the effects must be carefully monitored to avoid hyperglycemia, hypoglycemia, metabolic acidosis and ketosis.

Replacement of β-cells can be achieved with pancreatic transplants. Whole pancreas or pancreatic islets transplantation was a promising procedure for treating T1D patients some 20 years ago. A recent report suggests that the major problem is that transplanted islets over time lose their ability to function, with most recipients returning to using insulin[39]. In addition, risks of islet transplantation include risks that are associated with the transplant procedure, in particularly bleeding and blood clot formation, as well as the side effects of immunosuppressive drugs that transplant recipients receive in order to prevent the immune system from rejecting the transplanted islets[40]. These issues must be solved before islet transplantation becomes a conventional therapeutic option for T1D patients.

Autoantigen-based vaccines are another novel approach for T1D patients treatment. It has been shown that the onset of insulitis can be diminished in the non-obese diabetic (NOD) mouse type 1 diabetes model by inoculation with endogenous β-cell autoantigens. However, major improvements in autoantigen-based vaccination strategies came with linkage of the autoantigen to adjuvants. This vaccine strategy has shown increasing promise for an effective, safe, and persistent suppression of β-cell specific autoimmunity. Although multi-component vaccines have been shown to be highly effective in animals, clinical trials for suppression of β-cell autoimmunity in human diabetes patients remain to be accomplished[41].

People with T2D are generally treated with drugs that stimulate insulin production and secretion from the β-cells. A major disadvantage of these drugs, however, is that insulin

production and secretion is promoted regardless of the level of blood glucose. Thus, food intake must be balanced against the promotion of insulin production and secretion to avoid hypoglycemia or hyperglycemia.

In recent years several new agents have become available to treat T2D. These strategies include reducing insulin resistance using glitazones, reducing hepatic glucose production through biguanides, and limiting postprandial glucose absorption with α-glucosidase inhibitors[42]. Treatment with these agents is directed at lowering circulating blood glucose and inhibiting postprandial hyperglycemic spikes. However, the drop in hemoglobin A1c obtained by these newer agents is less than adequate, suggesting that they will not improve the long-term control of diabetes mellitus.

Most recently, glucagon-like peptide-1 (GLP-1), a hormone normally secreted by neuroendocrine cells of the gut in response to food, has been suggested as a new treatment for T2D. GLP-1 treatment has an advantage over insulin therapy because GLP-1 stimulates glucose-dependent insulin secretion and production by pancreatic beta cells, which turns off when blood glucose levels drop. GLP-1 promotes euglycemia by increasing insulin release and synthesis and inhibiting glucagon release. Recently it was shown that pancreatic α-cells are also capable of producing GLP-1 in response to signals secreted by injured β-cells in the islets. As GLP-1 also exerts pro-proliferative and cytoprotective (anti-apoptotic) actions on β-cells, and may stimulate β-cell neogenesis, potential benefit from GLP-1 treatment could be much higher than initially supposed[43]. Most of the above-mentioned treatments are directed at lowering blood glucose levels and treating the consequences, rather than causes of diabetes. Because a common feature of diabetes is a reduction in β-cell mass, therapeutic treatments and related therapeutics that control and promote β-cell growth and survival (as GLP-1) are highly desirable in the field of medical science.

The basis for a potential novel strategy for preserving β-cell viability and function in diabetes

A novel challenge in the treatment of diabetes is to find a means to preserve or enhance β-cell mass by stimulating the growth of new β-cells, prolonging the lifespan of present β-cells, or both[44]. Advances in β-cell research have recently illuminated the important roles that the chemokine (C-X-C motif) ligand 12 (CXCL12) and the nuclear enzyme poly(ADP-ribose) polymerase-1 (PARP-1) assume in preserving β-cell viability. Chemokine CXCL12 and its receptor CXCR4 were shown to be an obligatory component of pancreatic islet formation during embryogenesis[45] and pancreatic islet regeneration[46]. At the same time, it was reported that the CXCL12/CXCR4 axis exerts cytoprotective actions on islet β-cells and promotes their survival. The ability of chemokine CXCL12 to protect existing β-cells and stimulate β-cell regeneration has recently elevated it to a central position in potential strategies for diabetes treatment. Poly(ADP-ribose) polymerase-1 (PARP-1) was shown to be an important regulator of cell death and a key molecule in maintenance of cellular energy

homeostasis. For the last two decades, the role of PARP-1 activation in the process of β-cell death has been subject to extensive investigations *in vitro* and *in vivo*. The complete protection of rodents treated with PARP-1 inhibitors or of mice lacking the PARP-1 gene from chemically-induced diabetes is remarkable and has identified PARP-1 as an essential downstream executor of diabetes development in animal models[47]. The prominent role of PARP-1 in diabetes development is attributed to the direct protective effects of PARP-1 inhibition (whether genetic or pharmacological) on β-cell survival.

CXCL12 has an important pro-survival role in β-cells

The chemokines are a family of small (8–10 kD) secreted cytokines with more than 50 family members. Chemokines mediate their function through specific heptahelical transmembrane G protein-coupled receptors[48]. The binding of chemokines to their receptors triggers specific signal transduction pathways, leading to diverse biological responses, including calcium flux, chemotaxis, respiratory burst, degranulation, phagocytosis and lipid mediator synthesis[49]. CXCL12 or stromal derived factor-1 (SDF-1), is a chemokine originally cloned from a bone marrow-derived stromal cell line by Tashiro et al.[50]. It is constitutively expressed in a wide range of tissues, including the bone marrow, lymph nodes, liver, lung, brain, heart, kidney, thymus, stomach, and abundantly in the pancreas, spleen, ovary and small intestine[51]. Most chemokine receptors are promiscuous as each can bind with high affinity to multiple chemokine ligands, this being an essential quality of chemokines to fine tune specific chemokine-induced responses. However, the opposite holds true for CXCL12 as its receptor CXCR4 exhibits very high specificity for CXCL12[52]. It was believed that the CXCL12/CXCR4 ligand-receptor pair acts without crosstalk with other chemokines or receptors, however recent data shows that CXCL12 also binds to and activates chemokine receptor CXCR7[53]. CXCR4 is highly conserved across species and is expressed on a wide variety of cell types, including hematopoietic cells, vascular endothelial cells, neurons, microglia and astrocytes[51].

A major function of the CXCL12/CXCR4 axis is chemoattraction during leukocyte trafficking and stem cell homing during which local tissue gradients of CXCL12 attract circulating hematopoietic and tissue-committed somatic stem cells[54]. Also, the CXCL12/CXCR4 axis is critically involved in processes such as embryonal development[55], regulation of angiogenesis[56], regulation of HIV infection[57], cancer growth and metastasis[58]. Although a number of chemokines play critical roles in organogenesis, CXCL12 and CXCR4 comprise the only chemokine/chemokine receptor pair that individually results in late embryonic lethality in mouse knockouts[59]. Genetically deficient embryos display severe defects in their gastrointestinal vasculature, cerebellar neuron migration, cardiac ventricular septal closure, B cell development, and hematopoietic bone marrow colonization[60,61,62]. These defects highlight the important and conserved role of CXCL12 as a factor determiniong proper cell localization throughout the body. The CXCL12/CXCR4 axis is also involved in many aspects of cell survival and tissue repair and regeneration[63]. The importance of CXCL12 in stem and

progenitor cell recruitment has been established by observation that selective expression of CXCL12 in injured tissues correlates with adult stem cell recruitment and tissue regeneration[64]. The capability of CXCL12 to improve the process of tissue regeneration is of particular interest in diabetes where the irreversible loss of β-cell mass underlies the disease.

Regeneration of pancreatic β-cell mass after either toxin- or autoimmune-mediated destruction is possible in the young rodent, however, the extent of the recovery decreases with age and is incomplete in adult life[65]. Under specific conditions the renewal of β-cells occurs postnatally, while β-cells potentially originate from several sources. At the moment, there is evidence for an increase in β-cell mass through replication of existing β-cells, as well as via neogenesis from multipotent precursor cells residing in pancreatic ducts[65]. In the γ-interferon transgenic mouse, the model system of regenerating pancreas, it was shown that CXCL12 is an essential component in survival, proliferation and migration of CXCR4-expressing ductal epithelium cells in response to a CXCL12 gradient[45]. It is believed that the age-related loss of regenerative capacity after β-cell depletion is caused by a lack of one or more rate-limiting trophic stimuli but that it is potentially reversible[65]. CXCL12 might be the missing trophic factor as it is widely expressed in pancreatic cells, including β-cells, during embryogenesis, while in the adult pancreas CXCL12 expression is restricted to cells surrounding ducts and the microvasculature[66].

The prosurvival effect of CXCL12 observed upon binding to the CXCR4 receptor is another well documented feature of the CXCL12/CXCR4 axis. The CXCL12/CXCR4 pair has been shown to exert survival-enhancing effects on CD4+ T cells, dendritic cells, early cells of the T-lymphoid series, myeloid progenitor cells (MPC) in human peripheral blood and cord blood (CB), and MPC in mouse bone marrow[67,68]. Besides blood cells, treatment with CXCL12 protects INS-1 cells (a rat insulinoma cell line), against injury induced by serum withdrawal, thapsigargin, cytokines and glucotoxicity[44]. Also, transgenic mice expressing CXCL12 under the control of the insulin promoter in β-cells (RIP-SDF-1 mice) are protected against STZ-induced diabetes, suggesting that CXCL12 agonists could provide beneficial effects in the treatment of diabetes[69].

CXCL12 has been shown to exert its protective effects by activating the prosurvival phosphatidylinositol 3-kinases (PI3K)/Akt and extracellular-signal-regulated kinase (ERK) pathways[70]. Akt, also known as protein kinase B (PKB), is serine/threonine protein kinase that functions as a central node in cell signaling downstream of growth factors, cytokines, cell stressors and other stimuli that critically influence different cellular processes such as cell growth, survival, apoptosis and proliferation. Akt kinase enhances cell survival by blocking the function of proapoptotic proteins and thus inhibiting the apoptotic process. It has direct effects on the apoptotic pathway by targeting the proapoptotic Bcl-2 family member Bad whose phosphorylation completely inhibits its proapoptotic function[71]. In addition, Akt phosphorylates caspase-9, an initiator caspase involved in the intrinsic apoptotic pathway, which results in attenuation of its proteolytic activity[72]. Akt kinase also affects the transcriptional response to apoptotic stimuli through phosphorylation of transcription factors

forkhead box protein O (FOXO) and p53. Phosphorylation of FOXO by Akt displaces this *trans*-acting factor from target genes and triggers their export from nucleus. Through this mechanism, Akt blocks FOXO-mediated transcription of target genes that promote apoptosis, cell-cycle arrest and metabolic processes[73]. Another substrate for Akt is the murine double minute (MDM2), an ubiquitin ligase that triggers p53 degradation. Phosphorylation of MDM2 by Akt promotes translocation of MDM2 to the nucleus where it negatively regulates p53 function[74].

The above-mentioned beneficial effects of Akt were documented in pancreatic β-cells as well. In studies with Akt knockout mice and transgenic mice constitutively expressing active Akt in β-cells, Akt was shown to be a major regulator of β-cell mass[75,76]. Akt overexpression in β-cells leads to islet hyperplasia and resistance to streptozotocin (STZ)-induced apoptosis[77]. The enhancement of β-cell mass may be attributed to β-cell survival rather than to the proliferation of existing β-cells. It has been shown that the hormones like insulin-like growth factor 1 (IGF-1), insulin and glucagon-like peptide-1 (GLP-1) that regulate β-cell proliferation and survival, exert their effects via Akt activation in β-cells[69]. In addition, Yano et al. reported that CXCL12 promotes pancreatic β-cell survival through Akt activation[69].

ERK1/2 is another kinase downstream of the CXCL12/CXCR4 axis. The serine/threonine kinase(s) ERK1/2 belongs to the mitogen-activated protein kinase (MAP) kinase family. Although activation of ERK1/2 can promote apoptotic cell death as the other MAP kinases[78], the general consequence of ERK1/2 activation is promotion of cell survival, growth and differentiation[79]. ERK1/2 exerts its effects through downstream transcription factor targets that include NF-κB, cAMP response element-binding protein (CREB), Ets-1, AP-1 and c-Myc. These transcription factors induce the expression of genes important for cell cycle progression and proliferation (cyclins, Cdks, growth factors), and for apoptosis prevention and cell survival, by regulating the expression of antiapoptotic Bcl-2 and cytokines[80]. ERK1/2 is phosphorylated and activated via diverse signal transduction mechanisms initiated by phosphorylation of receptor tyrosin kinases (RTKs) or stimulation of G-protein-coupled receptors (GPCRs). Thus, binding of CXCL12 to the CXCR4 receptor induces activation of ERK1/2 in various cell types, from B and T-lymphocytes, neuronal cells to pancreatic exocrine and duct cells[45,81,82,83]. However, Yano et al. reported that treatment with the CXCR4 antagonist AMD3100 does not influence the ERK1/2 phosphorylation status in pancreatic β-cells (MIN6 cells), suggesting that CXCL12 exerts its prosurvival effects in β-cells primarily through activation of Akt[69].

According to a novel hypothesis, CXCL12 has the potential to enhance β-cell survival not only through antiapoptotic and autocrine actions, but also through a paracrine effect on adjacent α-cells in the islet of Langerhans[44]. It was shown that injured β-cells produce CXCL12 and that the subsequent activation of the CXCL12/CXCR4 axis in α-cells stimulates α-cell proliferation and switches its biological functions. Namely, α-cells that normally produce glucagon involved in glucose metabolism, after activation of the CXCL12/CXCR4 axis switch to providing local growth factors such as GLP-1 which is involved in the regenera-

tion of the injured β-cells[44]. It was previously documented that GLP-1 has antiapoptotic[85] and proliferative effect[43] on pancreatic β-cells. Therefore, in response to β-cell injury, the combination of CXCL12 secreted by β-cells and GLP-1 secreted by α-cell acts synergistically on β-cells to enhance their growth and survival[44]. The GLP-1 is already in use as a therapeutic agent in T2D patients because of its effect on glucose metabolism. Treatment with combination of GLP-1 and CXCL12 would not only stimulate insulin secretion, but also exert a direct effect on β-cells, enhancing β-cell mass and promoting their survival. Use of the CXCL12/GLP-1 combination is currently considered as a novel approach in treating diabetic patients.

…as well as PARP-1

PARP-1 is an abundant nuclear enzyme that catalyzes the polymerization of poly(ADP-ribose) (PAR) polymers on itself and other target proteins, including histones, DNA repair proteins and transcription factors. PARP-1 uses NAD^+ as a donor for the ADP-ribose unit whose polymerization results in linear or branched PAR polymers[85]. The catabolism of poly(ADP-ribose) polymers to free PAR or mono(ADP-ribose) is mediated by poly(ADP-ribose) glycohydrolase (PARG). PAR, as a negatively charged polymer, critically influences target or interacting proteins by modulating their function, structure and localization[86]. Poly(ADP-ribosyl)ation plays a variety of roles in many cellular processes, including DNA damage repair, chromatin modification, transcription and cell death. Until recently, PARP-1 was solely seen as DNA damage sensor as it was established that it can bind to both single- and double-strand breaks and that this interaction leads to activation of its enzymatic activity[85]. However, more recently it was shown that PARP-1 can also be activated in the absence of DNA damage by binding to nucleosomes or specific DNA structures, as well as by post-translational modifications[86,87].

Cells are constantly exposed to various extracellular and intracellular stress signals and the propagation of these signals involves crosstalk between many signaling pathways. Studies over the past years have placed PARP-1 in the junction of converging signaling pathways, recognizing it as a crucial determinant of cellular fate. Activation of PARP-1 and subsequent poly(ADP-ribosyl)ation of target proteins leads to immediate and dramatic biochemical responses, including chromatin remodeling, recruitment of the DNA repair machinery, modulation of activity of the transcriptional machinery and redistribution of the key regulatory proteins to different cellular compartments[88]. The specific role of PARP-1 in deciding cell fate depends on the type and intensity of stress signals and also on the metabolic and proliferative status of the cell. According to the prevailing view, a cell exposed to stress stimuli can enter different pathways depending on above mentioned circumstances. PARP-1 activation induced by mild or moderate stress signals leads to transcription and DNA repair that are aimed at maintaining genome stability and promoting cell survival without the risk of passing on mutated genes. In contrast to its role as a survival factor, PARP-1 acts to promote cell death in the presence of severe or sustained stress signals.

A cell can die through one of two cell death processes – apoptosis or necrosis. Apoptosis is a highly organized physiological process of cell death that is regulated by homeostatic mechanisms which allow for the safe elimination and disposal of cells. It is triggered and executed through an elaborate network of biochemical events and is energy dependent. Acute cell damage resulting from accidental exposure of cells to extreme physical damage, toxic insults and severe changes of physiological conditions induces a non-apoptotic type of cell death referred to as necrosis. Necrosis does not require the expenditure of cellular energy and is traditionally considered to be a disorganized process. However, evidence suggests that necrotic cell death can also be initiated from intracellular signals, i.e. that a cell can initiate its own death through the necrotic pathway[89]. PARP-1 is one of the mediators of so called programmed necrosis. In response to severe DNA damage, PARP-1 is activated very quickly which results in extensive auto- and poly(ADP-ribosyl)ation of target proteins. As PARP-1 uses NAD^+ as a substrate for this reaction, and NAD^+ is important cofactor in the process of glucolytic ATP synthesis, hyperproduction of PAR polymers leads to a severe depletion of cellular NAD^+ and ATP, with the ensuing energy failure resulting in necrotic cell death[90]. As apoptosis is an energy-dependent process, PARP-1 must be proteolytically inactivated early in this process in order to protect the cell from ATP depletion and necrosis, as well as unsuccessful attempts at DNA repair in a cell already 'foreseen' to die. During apoptosis, caspases-3 and -7 cleave PARP-1 into two fragments, separating the DNA binding and catalytic domains, thus resulting in inactivation of PARP-1 enzymatic activity[91]. Although PARP-1 activity seems to be dispensable for apoptotic process, PARP cleavage is necessary for appropriate function of the apoptotic machinery. Thus, PARP-1 serves as a molecular switch between apoptosis and necrosis, as its rapid activation can quickly prevail over the slower caspase-mediated apoptotic process. In addition, inhibiting or deleting PARP-1 which preserves cellular NAD^+ and ATP pools, results in an increase in apoptosis after exposure of cells to severe stress signals that would otherwise induce necrosis[92].

A critical role for PARP-1 in determining cell fate is confirmed by the observation that pharmacological inhibition or genetic deletion of PARP-1 protects cells or animals from pathophysiological conditions that lead to aberrant cell death, as seen in ischemia-reperfusion injury, glutamate excitotoxicity, cardiac infarction and inflammatory injury[93]. Moreover, PARP-1 knockout animals are protected against the development of insulin-dependent diabetes mellitus after exposure to the alkylating agent streptozotocin[94,95]. The same effect was achieved with PARP-1 pharmacological inhibitors, such 3-aminobenzamide (3-AB)[93]. The combined results from *in vivo* and *in vitro* studies using PARP-1 inhibitors indicate that protection against diabetes development is the result of a direct protection from necrotic cell death of β-cells. Namely, it was shown that treatment of β-cells or islets with a cytokine combination, ROS, nitric oxide (NO), hydrogen peroxide or STZ inhibits insulin secretion, induce PARP-1 activation, decrease in NAD+ and ATP, and results in cell death by necrosis[93]. Therefore, inhibition of PARP-1 activity and resulting energy failure is essential for the survival of β-cells and for the protection of animals from experimentally-induced diabetes[94]. It seems that PARP-1-mediated necrotic cell death is also relevant for the pathogenesis of

other diabetic complications, since treatment with PARP-1 inhibitors or genetically ablated PARP-1 alleviates, although it does not completely prevent, diabetic kidney disease, diabetic cardiomyopathy, diabetic microvascular complications and neuropathy[96,97,98]. PARP activation, thus, is a unique checkpoint in the development and progression of various diabetic complications.

Crosstalk between CXCL12 and PARP-1 promotes β-cell survival

Taking the earlier studies into consideration and the overall valuable effects of chemokine CXCL12 on pancreatic islets, our primarily goal is to explore the mechanisms involved in CXCL12-induced β-cell survival. As a model system we used the the Rin-5F rat insulinoma cell line: wild-type cells served as a control for stably transfected Rin-5F cells expressing human CXCL12. Ilhan *et al*. reported morphological difference between two cell lines[99]. The CXCL12-transfected cells mainly display a three-dimensional, cluster-like growth pattern, whereas the Rin-5F wild-type cells mostly display a two-dimensional, monolayer-like growth pattern. They demonstrated over-expression of human CXCL12 at the gene copy number and protein level. In CXCL12 transfectant clones, human CXCL12 gene copies exceeded the endogenous rat CXCL12 gene copy number 50–1000-fold. In wild-type Rin-5F cells, the endogenous rat CXCL12 copy number was comparable to the value detected in stable transfectants, whereas the human CXCL12 gene was absent. Supernatants taken from Rin-5F human CXCL12 transfectant clones contained 300–1000 pg/ml/10^6 cells/24 h of CXCL12, allowing for autocrine and paracrine CXCL12 signaling in stable transfectants, while the supernatants taken from wild-type cells were negative for the presence of this chemokine[99].

Our preliminary results suggest that Rin-5F cells overexpressing CXCL12 exhibit considerable resistance to both streptozotocin and hydrogen peroxide treatments in comparison to wild-type Rin-5F cells. According to the literature, the improved survival was most likely mediated by activation of Akt and/or ERK kinases that possess antiapoptotic and proliferative, prosurvival properties. However, it has been documented that β-cells can also undergo necrotic cell death in autoimmune attack or under glucolipotoxicity conditions. Considering the important role of PARP-1 in the induction of a necrotic type of cell death, we assumed that the prosurvival effects of CXCL12 could be at least in part mediated by modulation of PARP-1 activity. In agreement with our hypothesis, preliminary results indicate that CXCL12-overexpressing Rin-5F cells possess considerably lower PARP-1 activity in comparison with wild-type Rin-5F cells. Although pharmacological inhibition of PARP-1 improves β-cell viability, uncontrolled inhibition of an enzyme with so many important functions in the cell could contribute to genome instability. Therefore, an intrinsic mechanism of fine tuning PARP-1 activity would be a much more desirable mode in β-cell protection.

Recently, several new regulators of PARP-1 activity have been identified, including several kinases. Phosphorylation of PARP-1 can result in both augmentation and inhibition of its

enzymatic activity, depending on the kinase involved. Protein kinase C (PKC) and DNA-dependent protein kinase (DNA-PK) phosphorylate and inhibit PARP-1 activity, while PARP-1 phosphorylation mediated by 5' AMP-activated protein kinase (AMPK), ERK1/2 and c-Jun N-terminal kinase (JNK) was found to promote the catalytic activity of PARP-1[100]. We propose that some of the kinases downstream of the CXCL12/CXCR4 axis phosphorylate PARP-1 and lower its activity in CXCL12-overexpressing Rin-5F cells. Reduction of PARP-1 activity, in contrast to its inhibition, would allow repair of the damaged DNA and basic transcriptional activity in cells exposed to stress, but at the same time it would prevent PARP-1 overactivation, energy failure and necrotic cell death. The proposed modulation of PARP-1 activity by CXCL12 is a novel mechanism that would additionally explain the prosurvival effects of CXCL12.

Concluding remark

Diabetes mellitus remains an incurable disease. In view of the fact that β-cell dysfunction and cell loss is the central pathogenic mechanism in diabetes, it is our opinion that β-cells remain the prime target in the search for an effective diabetes treatment. Through an increased understanding of the cellular mechanisms and factors that control β-cell growth and survival, we may be able to glimpse a novel treatment for diabetes essentially based on augmenting β-cell mass. Aside from its potential to stimulate β-cell regeneration, its ability to prevent β-cells from entering the apoptotic process as well as to undergo necrotic cell death by modulating PARP-1 activity places chemokine CXCL12 in the focal point of developing strategies for diabetes treatment.

Acknowledgements

This work was supported by the Ministry of Education, Science and Tecnological Development of the Republic of Serbia, Grant No. 173020.

References

1. Hossain, P., Kawar, B. & El Nahas, M., (2007). Obesity and Diabetes in the Developing World — A Growing Challenge. *N. Engl. J. Med*., **356**, 213-215.
2. Cnop, M. *et al*., (2005). Mechanisms of pancreatic beta-cell death in type 1 and type 2 diabetes: many differences, few similarities. *Diabetes,* **54**, Suppl. 2, S97-107.
3. Gepts, W., (1965). Pathologic anatomy of the pancreas in juvenile diabetes mellitus. *Diabetes*, **14**, 619–633.
4. Bending, D., Zaccone, P. & Cooke, A., (2012). Inflammation and type one diabetes. *Int. Immunol*., **24**(6), 339-346.
5. Robertson, P. *et al*., (2003).Glucose Toxicity in β-Cells: Type 2 Diabetes, Good Radicals Gone Bad, and the Glutathione Connection. *Diabetes,* **52**, 581–587.

6. Robertson, P. *et al*., (2004). Beta-cell glucose toxicity, lipotoxicity, and chronic oxidative stress in type 2 diabetes. *Diabetes,* **53**(Suppl. 1), 119–124.
7. Poitout, V. & Robertson, P., (2008). Glucolipotoxicity: fuel excess and beta-cell dysfunction. *Endocr. Rev*., **29,** 351–366.
8. Robertson, R., (2004). Chronic Oxidative Stress as a Central Mechanism for Glucose Toxicity in Pancreatic Islet Beta Cells in Diabetes. *J. Biol. Chem,.* **279**, 42351–42354.
9. Robertson, P. & Harmon, J., (2006). Diabetes, glucose toxicity, and oxidative stress: A case of double jeopardy for the pancreatic islet β cell. *Free Rad. Bio.l Med.,* **41**, 177-184.
10. Wolff, S.P. & Dean, R.T., (1987). Glucose autoxidation and protein modification. The potential role of 'autoxidation glycosylation' in diabetes. *Biochem. J*., **245**, 243–259.
11. Lowell, B.B. & Shulman, G.I., (2005). Mitochondrial dysfunction and type 2 diabetes. *Science*, **307**, 384–387.
12. Morgan, D. *et al*., (2007). Glucose, palmitate and pro-inflammatory cytokines modulate production and activity of a phagocyte-like NADPH oxidase in rat pancreatic islets and a clonal beta cell line. *Diabetologia*, **50**, 359–369.
13. Ceriello, A., (1999). Hyperglycaemia: the bridge between non-enzymatic glycation and oxidative stress in the pathogenesis of diabetic complications. *Diabetes Nutr. Metab*., **12**, 42–46.
14. Kaneto, H. *et al*., (2001). Activation of the hexosamine pathway leads to deterioration of pancreatic beta-cell function through the induction of oxidative stress. *J. Biol. Chem*., **276,** 31099–31104.
15. Boucher, M.J. *et al*., (2006). Phosphorylation marks IPF1/PDX1 protein for degradation by glycogen synthase kinase 3- dependent mechanisms. *J. Biol. Chem*., **281**, 6395–6403.
16. Harmon, J.S., Stein, R. & Robertson, R.P., (2005). Oxidative stress-mediated, posttranslational loss of MafA protein as a contributing mechanism to loss of insulin gene expression in glucotoxic beta cells. *J. Biol. Chem*., **280**, 11107–11113.
17. Sachdeva, M.M. *et al*., (2009). Pdx1 (MODY4) regulates pancreatic beta cell susceptibility to ER stress. *Proc. Natl. Acad. Sci*., **106**, 19090–19095.
18. Kulkarni, R.N. *et al*., (2004). PDX-1 haploinsufficiency limits the compensatory islet hyperplasia that occurs in response to insulin resistance. *J. Clin. Invest*., **114**, 828–836.
19. Thomas, H.E. *et al*., (2009). Beta cell apoptosis in diabetes. *Apoptosis*, **14**, 1389–1404.
20. Federici, M. *et al*., (2001). High glucose causes apoptosis in cultured human pancreatic islets of Langerhans: a potential role for regulation of specific Bcl family genes toward an apoptotic cell death program. *Diabetes*, **50**, 1290–1301.
21. Gurgul, E. *et al*., (2004). Mitochondrial catalase overexpression protects insulin-producing cells against toxicity of reactive oxygen species and proinflammatory cytokines. *Diabetes*, **53**, 2271–2280.
22. Tiedge, M. *et al*., (1997) Relation between antioxidant enzyme gene expression and antioxidative defense status of insulinproducing cells. *Diabetes*, **46**, 1733–1742.
23. Pi, J. & Collins, S., (2010). Reactive oxygen species and uncoupling protein 2 in pancreatic β-cell function. *Diabetes Obes. Metab.,* **12** (Suppl. 2), 141–148.
24. Lenzen, S., (2008). Oxidative stress: the vulnerable β-cell. *Biochem. Soc. Trans.,* **36**, 343–347.
25. Henquin, J.C., (2000). Triggering and amplifying pathways of regulation of insulin secretion by glucose. *Diabetes*, **49**, 1751-1760.
26. Wollheim, C.B. & Maechler, P., (2002). Beta-cell mitochondria and insulin secretion: messenger role of nucleotides and metabolites. *Diabetes*, **51** (Suppl. 1), S37-S42.

27. Morgan, D., *et al*., (2009). Association of NAD(P)H Oxidase with Glucose-Induced Insulin Secretion by Pancreatic β-Cells. *Endocrinology*, **150**, 2197-2201.
28. Janjic, D. *et al*., (1999). Free Radical Modulation of Insulin Release in INS-1 Cells Exposed to Alloxan. *Biochem. Pharmacol.,* **57**, 639–648.
29. Pi, J. *et al*., (2008). Activation of Nrf2-mediated oxidative stress response in macrophages by hypochlorous acid. *Toxicol. Appl. Pharmacol*., **226**, 236-243.
30. Pi, J. *et al*., (2007). Reactive oxygen species as a signal in glucose-stimulated insulin secretion. *Diabetes*, **56**, 1783-1791.
31. Xu, B., Moritz, J & Epstein, P., (1999). Overexpression of catalase provides partial protection to transgenic mouse beta cells. *Free Rad. Biol. Med*., **27**, 830–837.
32. Benhamou, P.Y. *et al*, (1998). Adenovirus-mediated catalase gene transfer reduces oxidant stress in human, porcine and rat pancreatic islets *Diabetologia,* **41***,* 1093-1100.
33. Moriscot, C. *et al*., (2000). Contribution of adenoviral-mediated superoxide dismutase gene transfer to the reduction in nitric oxide-induced cytotoxicity on human islets and INS-1 insulin-secreting cells. *Diabetologia,* **43**, 625–631.
34. Hohmeier, H.E. *et al*., (1998). Stable expression of manganese superoxide dismutase (MnSOD) in insulinoma cells prevents IL-1beta- induced cytotoxicity and reduces nitric oxide production. *J. Clin. Invest*., **101**, 1811-1820.
35. Forman, H.J. & Torres, M., (2002). Reactive oxygen species and cell signaling: respiratory burst in macrophage signaling. *Am. J. Respir. Crit. Care Med*., **166**, S4–S8.
36. McClung, J.P. *et al*., (2004). Development of insulin resistance and obesity in mice overexpressing cellular glutathione peroxidase. *Proc. Natl. Acad. Sci.,* **101**, 8852–8857.
37. Li, X., Chen, H. & Epstein, P.N., (2006). Metallothionein and Catalase Sensitize to Diabetes in Nonobese Diabetic Mice: Reactive Oxygen Species May Have a Protective Role in Pancreatic beta-Cells. *Diabetes*, **55**, 1592–1604.
38. Wiernsperger, N.F., (2003). Oxidative stress as a therapeutic target in diabetes: revisiting the controversy. *Diabetes Metab*., **29**, 579–585.
39. Ryan, E.A. *et al*., (2005). Five-year follow-up after clinical islet transplantation. *Diabetes*, **54**, 2060-2069.
40. Robertson, R.P., (2004). Islet Transplantation as a Treatment for Diabetes — A Work in Progress. *N. Engl. J. Med*., **350**, 694-705.
41. Harrison, L.C., (2008). Vaccination against self to prevent autoimmune disease: the type 1 diabetes model. *Immunol. Cell Biol*., **86**, 139-145.
42. Wagman, A.S. & Nuss, J.M., (2001). Current therapies and emerging targets for the treatment of diabetes. *Curr. Pharm. Des*., **7**, 417-50.
43. Buteau, J. *et al*., (1999). Glucagon-like peptide-1 promotes DNA synthesis, activates phosphatidylinositol 3-kinase and increases transcription factor pancreatic and duodenal homeobox gene 1 (PDX-1) DNA binding activity in beta (INS-1)-cells. *Diabetologia*, **42**, 856-864.
44. Liu, Z. *et al*., (2011). Stromal cell-derived factor-1 (SDF-1)/chemokine (C-X-C motif) receptor 4 (CXCR4) axis activation induces intra-islet glucagon-like peptide-1 (GLP-1) production and enhances beta cell survival. *Diabetologia*, **54**, 2067-76.
45. Kayali, A.G. *et al*., (2012). The SDF-1α/CXCR4 Axis is Required for Proliferation and Maturation of Human Fetal Pancreatic Endocrine Progenitor Cells. *PLoS ONE*, **7**, e38721.
46. Ayse, G. *et al*., (2003). The stromal cell–derived factor-1α/CXCR4 ligand–receptor axis is critical for progenitor survival and migration in the pancreas. *J. Cell Biol*., **163**, 859–869.

47. Virag, L. & Szabo, C., (2002). The Therapeutic Potential of Poly(ADP-Ribose) Polymerase Inhibitors. *Pharmacol. Rev.,* **54**, 375–429.
48. Pierce, K.L., Premont, R.T. & Lefkowitz, R.J., (2002). Seven-transmembrane receptors. *Nat. Rev. Mol. Cell Biol*., **3**, 639–650.
49. Horuk, R., (2001). Chemokine receptors. *Cytokine Growth Fact. Rev*., **12**, 313-335.
50. Tashiro, K. *et al*.,(1993). Signal sequence trap: a cloning strategy for secreted proteins and type I membrane proteins. *Science*, **261**, 600-603.
51. Juarez, J., Bendall, L. & Bradstock, K., (2004). Chemokines and their Receptors as Therapeutic Targets: The Role of the SDF-1/CXCR4 Axis. *Curr. Pharm. Design*, **10**, 1245-1259.
52. Nagasawa, T. *et al*., (1996). Molecular cloning and characterization of a murine pre-B-cell growth-stimulating factor/stromal cell-derived factor 1 receptor, a murine homolog of the human immunodeficiency virus 1 entry coreceptor fusin. *Proc. Natl. Acad. Sci*., **93**, 14726-14729.
53. Burns, J.M. *et al*., (2006). A novel chemokine receptor for SDF-1 and I-TAC involved in cell survival, cell adhesion, and tumor development. *J. Exp. Med*., **203**, 2201–2213.
54. Aiuti, A. *et al*., (1997). The chemokine SDF-1 is a chemoattractant for human CD34 hematopoietic progenitor cells and provides a new mechanism to explain the mobilization of CD34 progenitors to peripheral blood. *J. Exp. Med*., **185**,111–120.
55. McGrath, K.E. *et al*., (1999). Embryonic expression and function of the chemokine SDF-1 and its receptor, CXCR4. *Dev. Biol*., **213**, 442-456.
56. Ho, T.K. *et al*., (2012). Stromal-Cell-Derived Factor-1 (SDF-1)/CXCL12 as Potential Target of Therapeutic Angiogenesis in Critical Leg Ischaemia. *Card. Res. Practice*, **2012**, 1-7.
57. Feng, Y. *et al*., (1996). HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane G protein-coupled receptor. *Science*, **272**, 872–877.
58. Kryczek, I. *et al*., (2007). Stroma-derived factor (SDF-1/CXCL12) and human tumor pathogenesis. *Am. J. Physiol. Cell Physiol*., **292**, C987–C995.
59. Murphy, P.M. *et al*., (2000). International union of pharmacology. XXII. Nomenclature for chemokine receptors. *Pharmacol. Rev*., **52**, 145–176.
60. Ma, Q. *et al*., (1998). Impaired B-lymphopoiesis, myelopoiesis, and derailed cerebellar neuron migration in CXCR4- and SDF-1-deficient mice. *Proc. Natl. Acad. Sci*., **95**, 9448–9453.
61. Tachibana, K. *et al*., (1998). The chemokine receptor CXCR4 is essential for vascularization of the gastrointestinal tract. *Nature*, **393**, 591–594.
62. Zou, Y.R. *et al*., (1998). Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. *Nature*, **393**, 595–599.
63. Ratajczak, M.Z. *et al*., (2006). The pleiotropic effects of the SDF-1-CXCR4 axis in organogenesis, regeneration and tumorigenesis. *Leukemia*, **20**, 1915–1924.
64. Schober, A. *et al*., (2006). SDF-1alpha-mediated tissue repair by stem cells: a promising tool in cardiovascular medicine? *Trends Cardiovasc. Med*., **16**, 103–112.
65. Thyssen, S., Arany, E. & Hill, D.J., (2006). Ontogeny of Regeneration of β-Cells in the Neonatal Rat after Treatment with Streptozotocin. *Endocrinology*, **147**, 2346–2356.
66. Hick, A.C. *et al*., (2009). Mechanism of primitive duct formation in the pancreas and submandibular glands: a role for SDF-1. *BMC Dev. Biol*., **9**, 66.
67. Broxmeyer, H.E. *et al*., (2003). Stromal cell-derived factor-1/ CXCL12 directly enhances survival/ antiapoptosis of myeloid progenitor cells through CXCR4 and G(alpha)i proteins and enhances engraftment of competitive, repopulating stem cells. *J. Leukoc. Biol*., **73**, 630–638.

68. Guo, Y., *et al*., (2005). SDF-1/CXCL12 Enhances Survival and Chemotaxis of Murine Embryonic Stem Cells and Production of Primitive and Definitive Hematopoietic Progenitor Cells. *Stem Cells*, **23**, 1324–1332.
69. Yano, T. *et al*., (2007). Stromal cell derived factor-1 (SDF-1)/CXCL12 attenuates diabetes in mice and promotes pancreatic beta-cell survival by activation of the prosurvival kinase Akt. *Diabetes*, **56**, 2946–2957.
70. Majka, M. *et al*., (2000). Binding of stromal derived factor-1alpha (SDF-1alpha) to CXCR4 chemokine receptor in normal human megakaryoblasts but not in platelets induces phosphorylation of mitogen-activated protein kinase p42/44 (MAPK), ELK-1 transcription factor and serine/threonine kinase AKT. *Eur. J. Haematol*., **64**, 164–172.
71. Datta, S.R. *et al*., (1997). Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery, *Cell*, **91**, 231-241.
72. Cardone, M.H. *et al*., (1998). Regulation of cell death protease caspase-9 by phosphorylation, *Science*, **282**, 1318-1321.
73. Burgering, B.M. & Medema, R.H., (2003). Decisions on life and death: FOXO Forkhead transcription factors are in command when Akt/PKB is off duty. *J. Leukoc. Biol*., **73**, 689-701.
74. Gottlieb, T.M. *et al*., (2002). Cross-talk between Akt, p53 and Mdm2: possible implications for the regulation of apoptosis, *Oncogene*, **21**, 1299-1303.
75. Tuttle, R.L. *et al*., (2001). Regulation of pancreatic beta-cell growth and survival by the serine/threonine protein kinase Akt1/PKBalpha. *Nat. Med*., **7**, 1133–1137.
76. Garofalo, R.S. *et al*., (2003). Severe diabetes, age-dependent loss of adipose tissue, and mild growth deficiency in mice lacking Akt2/PKB beta. *J. Clin. Invest*., **112**, 197–208.
77. Bernal-Mizrachi, E. *et al*., (2001). Islet beta cell expression of constitutively active Akt1/PKB alpha induces striking hypertrophy, hyperplasia, and hyperinsulinemia. *J. Clin. Invest*., **108**, 1631–1638.
78. Chu, C.T. *et al*., (2004). Oxidative neuronal injury. The dark side of ERK1/2. *Eur. J. Biochem*., **271**, 2060-2066.
79. Xia, Z. *et al*., (1995). Opposing Effects of ERK and JNK-p38 MAP Kinases on Apoptosis. *Science*, **270**, 1326-1331.
80. Chang, F. *et al*., (2003). Signal transduction mediated by the Ras/Raf/MEK/ERK pathway from cytokine receptors to transcription factors: potential targeting for therapeutic intervention. *Leukemia*, **17**, 1263-1293.
81. Shen, X. *et al*., (2010). Chemokine Receptor CXCR4 Enhances Proliferation in Pancreatic Cancer Cells Through AKT and ERK Dependent Pathways. *Pancreas*, **39**, 81-87.
82. Tilton, B. *et al*., (2000). Signal Transduction by CXC Chemokine Receptor 4: Stromal Cell–derived Factor 1 Stimulates Prolonged Protein Kinase B and Extracellular Signal–regulated Kinase 2 Activation in T Lymphocytes. *J. Exp. Med*., **192**, 313–324.
83. Luo, Y. *et al*., (2008). SDF1α/CXCR4 Signaling, via ERKs and the Transcription Factor Egr1, Induces Expression of a 67-kDa Form of Glutamic Acid Decarboxylase in Embryonic Hippocampal Neurons. *J. Biol. Chem*., **283**, 24789–24800.
84. Yusta, B. *et al*., (2006). GLP-1 receptor activation improves B cell function and survival following induction of endoplasmic reticulum stress. *Cell Metab*., **4**(5), 391-406.
85. D'Amours, D. *et al*., (1999). Poly(ADP-ribosyl)ation reactions in the regulation of nuclear functions. *Biochem. J*., **342**, 249–268.

86. Krishnakumar, R. & Kraus, W.L., (2010). The PARP side of the nucleus: Molecular actions, physiological outcomes, and clinical targets. *Mol. Cell*, **39**, 8–24.
87. Kim, M.Y. *et al*., (2004). NAD+-dependent modulation of chromatin structure and transcription by nucleosome binding properties of PARP-1. *Cell*, **119**, 803–814.
88. Luo, X. & Kraus, W.L., (2012). On PAR with PARP: cellular stress signaling through poly(ADP-ribose) and PARP-1. *Genes Dev*., **26**, 417–432.
89. Zong, W.X. *et al*., (2004). Alkylating DNA damage stimulates a regulated form of necrotic cell death. *Genes Dev*., **18**, 1272–1282.
90. Ha, H.C. & Snyder, S., (1999). Poly(ADP-ribose) polymerase is a mediator of necrotic cell death by ATP depletion. *Proc. Natl. Acad. Sci*., **96**, 13978–13982.
91. D'Amours, D. *et al*., (2001). Gain-of-function of poly(ADP-ribose) polymerase-1 upon cleavage by apoptotic proteases: implications for apoptosis. *J. Cell Sci*., **114**, 3771–3778.
92. Palomba, L. *et al*., (1996). Prevention of necrosis and activation of apoptosis in oxidatively injured human myeloid leukemia U937 cells. *FEBS Lett*., **390**, 91–94.
93. Virag, L. & Szabo, C., (2002). The Therapeutic Potential of Poly(ADP-Ribose) Polymerase Inhibitors. *Pharmacol. Rev*., **54**, 375–429.
94. Burkart, V. *et al*., (1999). Mice lacking the poly(ADP-ribose) polymerase gene are resistant to pancreatic beta-cell destruction and diabetes development induced by streptozocin. *Nat. Med*., **5**, 314–319.
95. Pieper, A.A. *et al*., (1999). Poly-ADP-ribose polymerase-deficient mice are protected from streptozotocin-induced diabetes. *Proc. Natl. Acad. Sci*., **96**, 3059–3064.
96. Choi, S.K. *et al*., (2012). Poly(ADP-Ribose) Polymerase 1 Inhibition Improves Coronary Arteriole Function in Type 2 Diabetes Mellitus. *Hypertension*, **59**, 1060-1068.
97. Shevalye, H. *et al*., (2010). Poly(ADP-ribose) polymerase-1 (PARP-1) gene deficiency alleviates diabetic kidney disease. *Biochim. Biophys. Acta*, 1802(**11**), 1020-1027.
98. Kuchmerovska, T. *et al*., (2004). Poly(ADP-ribosyl)ation enhancement in brain cell nuclei is associated with diabetic neuropathy. *J. Diabetes Complications,* **18**, 198-204.
99. Ilhan, A. *et al*., (2009). CXCL12/SDF-1 over-expression in human insulinomas and its biological relevance. *Mol. Cell Endocrin*., **298**, 1–10.
100. Zhang, S. *et al*., (2007). C-Jun N-terminal kinase mediates hydrogen peroxide-induced cell death via sustained poly(ADP-ribose)polymerase-1 activation. *Cell Death Differ*., **14**, 1001-1010.

Genetic basis of inflammation in human diseases

Aleksandra Stanković[1*], Maja Živković[1]

*[*1] Laboratory for Radiobiology and Molecular Genetics Institute of Nuclear Sciences "Vinča", University of Belgrade, Belgrade, Serbia*
e-mail: alexas@vinca.rs

What do heart disease, diabetes, neurodegenerative disease, stroke and cancer have in common? Chronic inflammation-the long-term inflammatory processes directed at a particular endogenous or exogenous antigen. It has been estimated that inflammatory biomarkers have a strong predicted genetic component. The idea is to define genetic markers from the various biological pathways (cytokines, chemokines, growth factors, bioactive molecules, endogenous proteases, regulatory RNA) that could affect and modulate inflammation and response to inflammatory stimuli. The aim is to establish a link between genetic susceptibility to various diseases and the response to inflammatory signaling, and to examine and demonstrate the utility of gene or genome-wide association studies findings in improving disease prevention by inclusion of genetic risk factors in calculation of the disease risk score. During this process, the new molecular mechanisms that underlie inflammation, regulation and pathological changes in human diseases have become known.

Molecular Basis of Chronic inflammation

Chronic inflammatory diseases are defined by long-term inflammatory processes directed at a particular endogenous or exogenous antigen. Within this definition, there is considerable overlap with autoimmune conditions such as multiple sclerosis, but it can also be extended to cover a range of other conditions that would not normally be termed 'autoimmune', such as, atherosclerosis. However, atherosclerosis also may be viewed as a chronic autoimmune disease with adaptive immune responses to atherosclerosis-associated antigens.

Inflammation is an important component of host defense; however, excessive or uncontrolled inflammation can disrupt normal tissue homeostasis. Traumatic injury can produce a massive, systemic inflammatory response that complicates resuscitation and patient outcomes. In addition, smaller, chronic tissue insults that occur during disease formation, such as long-term infectious agents, hyperlipidemia, hyperglycemia, ischemic injuries, elevated oxidative stress, etc., can alter the expression of inflammatory mediators and receptors, as well as intracellular signal transduction pathways and structural components in the target tissue (vascular endothelium, blood brain epithelium, renal epithelium, middle ear epithelium)

facilitating exaggerated inflammatory responses or a chronic inflammatory state. However, the stable presence of activated monocyte–macrophage cells in the target tissue is pathogenic. Key point is that endothelial cell (in the vessel wall/in blood brain barrier) and epithelial cells (in the kidney/middle ear) become disrupted or activated, when growth factors and degrading proteases are released from infiltrating mononuclear cells monocite/macrophage or resident macrophages of the brain and spinal cord (microglia cells that are a type of glial cell in CNS) or activated fibroblasts.

Pathological features of both atherosclerosis and multiple sclerosis are disruption of endothelium (blood vessel endothelium or blood brain barrier endothelium) formation of inflammatory lesions (plaque) with inflammatory infiltrates that mainly consist of lymphocytes and macrophages (in particular monocyte-derived macrophages)[1,2]. Blood-derived inflammatory cells participate in and perpetuate a local inflammatory response. Blood leukocytes, mediators of host defenses and inflammation, localize in the earliest lesions both in brain and blood vessel and the first phase of both diseases is defined as inflammatory phase. Cytokines and vasoactive peptides, such as tumor necrosis factor (TNF)-a, angiotensin (Ang) II, can induce the expression of adhesion molecules, which causes leukocytes rolling along endothelial cells to adhere at the site of activation[3-5] stimulate macrophages to “eat” lipids (ox LDL, or myelin) and form inflammatory cores (that perpetuate inflammation) and finally tissue damage lesions.

Pathological changes and proinflammatory cascade become extended by production not only cytokines and growth factors but also extracellular proteases, e.g. matrix metalloproteinases (MMPs). MMP production by monocyte/macrophages is dependent on the cell type, state of differentiation, and/or level of activation. The MMPs play an important role in tissue remodeling associated with various physiological and pathological processes. They are known to be involved in the cleavage of cell surface receptors, the release of apoptotic ligands (such as the FAS ligand), and chemokine/cytokine in/activation[6]. MMPs are also thought to play a major role on cell behaviors such as cell proliferation, migration (adhesion/dispersion), differentiation, angiogenesis, apoptosis, and host defense. They are suggested to play a role in the influx of inflammatory cells into the CNS, disruption of the BBB, and have been shown to degrade myelin in vitro[7-9]. In atherosclerosis they play an important role in extracellular matrix remodeling, cell migration and plaque remodeling and rupture[10, 11]

Heritability of inflammatory biomarkers

It has been estimated by twin studies that inflammatory biomarkers have a strong predicted genetic component[12]. As genetic effects are fixed during lifetime the idea was to define heritable inflammatory characteristics in humans, to assess lifelong inflammatory status more accurately. During the last decade the enormous quantity of data indicated that inflammatory molecules and pathways plays important role in different end organ damage. In this paper we will focus on the genetics of inflammatory condition in a subset of inflammatory diseases:

within cardiovascular system (atherosclerosis) and within central nervous system (multiple sclerosis), to define genetic markers of these conditions.

The genetic variations in determinants of inflammatory pathways can significantly influence the susceptibly and progression to certain steps in the inflammatory cascade thus, the susceptibility and progression of the disease itself. The atherosclerosis and multiple sclerosis are the classical examples of the polygenic complex disease, with strong inflammatory background caused by both genetic and environmental factors.

Genetic epidemiology approach in investigation of human complex diseases

Determination of genetic risk factors for susceptibility and progression of human complex diseases represents a main challenge for modern medicine and molecular genetics. In last few years the great breakthrough in molecular genetics methodology, as well as in new diagnostic and imaging procedures made complete workflow of investigation of such diseases more accurate and efficient. The more precise estimation of health risk and the efficiency of therapy, on our way toward personalized medicine, are ultimate goals of molecular medicine.

Genetic epidemiology represents the study of the role of genetic factors in determining health and disease in families and in populations, and the interactions of such genetic factors with environmental factors.
Since the discovery of PCR (polymerase chain reaction)[13, 14] for which is Nobel Prize were given to Karl Mullis in 1993., and its application in amplification of DNA fragments by thermostable polymerase[15] it became possible to widely investigate the association of differences in DNA primary structure with disease development. It changed the focus of the research and the variations in genetic structure and its association with disease become one of the primary goals in analysis of multifactorial polygenic diseases. Demographic and population differences set as necessity investigations in different populations and confirmational studies for the results obtained in the other ethnical or demographic group.

The markers of genetic variability, gene polymorphisms are the main genetic epidemiology tool in association studies. It is estimated that in human genome there is about 10 millions of single nucleotide polymorphisms (SNP) with minor allele frequency >1%[16,17], which represents more than 90% of human genome variability.

The concept of the association studies requires determination of the genetic variation (SNP) and its minor allele frequency in the healthy control population of the same ethnic origin as study group. The next step is detection of the same polymorphism variant in the group of patients from the same population and investigation of the association with disease. The measure of the strength of association between genotypes and/or haplotypes and disease, in this review it is atherosclerosis is expressed in terms of OR and 95% confidence interval (CI). Multiple regression analysis is usually used to determine the influence of modifying factors on susceptibility to complex, multifactorial diseases. Association studies of this type

have good prospects for dissecting the genetics of common disease, but they currently face a number of challenges, including problems with multiple testing and study design, definition of intermediate phenotypes and interaction between polymorphisms. Virtually all the association studies have been performed through hypothesis-driven research and candidate gene approaches. On the other hand, the trend in human complex disease genetics is shifting more and more towards hypothesis-generating genome-wide association studies (GWAS). GWAS for common diseases have been successful at identifying novel SNP variants in unexpected genes, but they have come up mostly with common variants of modest effects, which cannot explain more than a minimal fraction of heritability.

The final aim of all type of genetic association studies is to improve disease prevention by inclusion of genetic risk factors in calculation of the disease risk score.

Chemokines and Citokines

Adhesion of leukocytes to endothelial cells, either in blood vessel or blood brain barrier, and their subsequent transmigration are important steps in the initiation of the inflammatory process leading to the development of tissue damage. The monocyte chemoattractant molecule (MCP-1 or CCL2), fraktalkine (CX3CL1), CXCL16 and P-selectin ligand (PSLG-1) are among the most important molecules included in the first inflammatory events in e.g. atherosclerosis: monocyte recruitment and rolling, adhesion to endothelium and infiltration to vascular wall.

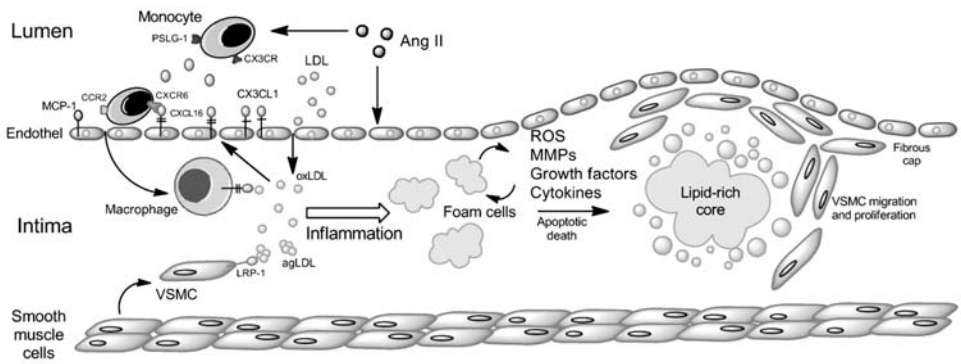


Figure 1. Key events and molecules involved in inflammatory cascade in atherosclerosis.

It has been reported that polymorphism A/G at position –2518 upstream the major transcription site of the MCP-1 gene, influence the promoter activity and level of MCP-1 gene expression in response to an inflammatory stimulus in vitro, and correlates with individual differences in monocyte MCP-1 production in dose dependent manner[18]. This polymorphism is

the most investigated one in the gene for MCP-1, not only in atherosclerosis but in various inflammatory diseases. In the study in patients with carotid atherosclerosis in Serbia, we didn't find its association with susceptibility to disease, but the mRNA expression in atherosclerotic plaque tissue was abundantly present and significantly higher compared to the control arteries[19]. The results of other studies also point out the association of polymorphism in MCP-1 with circulatory levels of the protein and the gene expression[18,20,21], rather that with disease susceptibility. Recent findings confirmed the previously suggested therapheutic potential of MCP-1 in atherosclerosis[22] by showing the –2518A/G allele dependent response to reductions in high-sensitivity C-reactive protein levels[23].

Among the number of chemokines we chose to discuss two of them with very unique functional properties, fraktakline and CXCL16. They act as both chemokine and adhesion molecules[24-26], and each of them interact with its' single chemokine receptor (CX3CR1 and CXCR6, respectively).

The most interesting genetic variations in these chemokine/receptor complexes represent the variants that are implicated in the alterations in receptor binding affinity or adhesion process. These are SNPs V249I (rs3732379) and T280M (rs3732378) in fraktalkine receptor coding sequence[27] and SNPs I123T (rs1050998) and A181V (rs2277680) in exon 4 of the CXCL16 gene[28]. The SNPs in fraktalkine receptor are studied previously, mostly in coronary artery disease[29], but the studies about CXCL16 gene variations are scarce.

One of the ways to bioinformaticaly predict of the effects that variations in the DNA could have on ligand/receptor interaction is by the informational spectrum method (ISM) as an established sequence analysis method[30]. Our group used this method to predict the effects that CX3CR1 sequence variations (V249I and T280M) could have on CX3CR1-CX3CL1 interaction and CXCL16 sequence variations (I123T and A181V) could have on CXCR6-CXCL16 interaction. The ISM analysis of CX3CR1 protein sequence variations' revealed that, out of three common protein (haplotype) variants, the I249 T280 protein variant could have significantly different interaction with CX3CL1 in comparison to wild-type, V249 T280. The same haplotype was found to be significantly protective for switch to secondary progressive form of multiple sclerosis[31]. The ISM analysis showed significantly different effects of CXCL16 haplotypes I123A181 and T123V181, and opposite effects of the sole rare alleles, T123 and V181 on CXCL16-CXCR6 interaction[32]. These results should be experimentally confirmed which is under ongoing study in both patients with multiple sclerosis[33] and atherosclerosis[34]. Still, previous functional study revealed that CXCL16 protein variants corresponding to these two common haplotypes differed by presence/absence of adhesive property[35].

Another major molecule that mediates leukocyte-endothelium and platelet-endothelial interactions[36-38] and might contribute to thrombosis/inflammation is P-selectin ligand (PSGL-1), the counter-receptor for P-selectin on myeloid cells and stimulated T lymphocytes. The coding genetic variant Met62Ile in the PSGL-1 gene was independently associated with PSGL-1 levels. The carriers of haplotype containing Ile allele had significantly increased levels of

PSGL-1[39]. Recently, our group found that the Ile/Ile homozygotes had significantly higher CRP levels compared to the other genotypes as well as the sex-differential association of Met62Ile with advanced carotid atherosclerosis (female patients had Ile allele dose-dependent association with the carotid plaque presence)[40]. It is conceivable that the genotype associated with higher PSGL-1 levels could be associated with higher inflammatory parameters, such as CRP. Also, there is a possibility of the linkage of Met62Ile polymorphism with another polymorphism, which influence CRP levels. The CRP, is a systemic marker of inflammation that predicts future cardiovascular risk[41,42]. It is interesting that family and twin studies suggest that additive genetic factors that account for the variance in plasma CRP levels are different in Europeans, from 25%–40%[43-45] and in African-Americans, about 45%[46].

Recent large genome-wide association studies (GWASs) conducted on Europeans and Japanese have identified approximately 20 CRP-associated genetic[47-50]. Many of these genes involve pathways related to innate immunity (these are CRP, IL6R, IL6, NLRP3, IL1RN). Many of them showed consistent patterns of association with CRP in African-American and Hispano-American women, but the new locus have been identified as associated with CRP in United States minority populations, a common TREM2 variant. This is a nice example of a genetic variant associated with certain phenotype but specific to populations of particular descent, e.g. African[51].

Most of the CRP as well as fibrinogen production is driven by interleukin 6 (IL-6) the pleiotropic cytokine, which is the major inducer of acute-phase response. The IL-6 protein levels are highly heritable and may be under significant genetic influence[52], although several factors may confound this relationship, including physical activity and metabolic alterations[53]. The IL6 gene has a number of common SNPs; the most studied is the G to C base change at position -174 in the promoter region[54]. We didn't find the association of this polymorphism with onset of either carotid atherosclerosis (our unpublished data) or kidney damage after acute infections in children[55]. Recent data suggest that blockade of IL-6 receptor (IL6R), which is expressed on monocytes, hepatocytes, and endothelial cells [56], actually has anti inflammatory actions, that extend beyond reductions in C-reactive protein and fibrinogen concentrations[57,58]. On the basis of most recent genetic evidence in humans, IL6R signaling seems to have a causal role in development of coronary heart disease. The IL6R SNP (rs7529229) was associated with increased circulating log interleukin-6 concentration, reduced C-reactive protein and fibrinogen concentrations as well as decreased odds of coronary heart disease events[59]. IL6R blockade could provide a novel therapeutic approach to prevention of coronary heart disease as well as similar inflammatory disease.

Renin-Angiotensin System (RAS)

The special place in inflammatory actions belongs to Angiotensin II (Ang II), the main peptide hormone of the renin–angiotensin system (RAS), which acts through the AT1 receptor (AT1R) and AT2 receptor (AT2R). It is likely to act as an inflammatory mediator through different mechanisms. It has been shown to have a proinflammatory effect on leukocytes,

endothelial cells, and vascular smooth muscle cells[60], and is well known to induce leukocyte recruitment to the vessel wall, which is a hallmark of the early stages of atherosclerosis and several hypertensive diseases[61]. Also, Ang II causes endothelial dysfunction by inducing cytokine and chemokine secretion. AT1 receptors for Ang II have recently been found on circulating neutrophils[62]. The renin-angiotensin system could serve an important role in promoting inflammation[63-64]. Recently it has been shown that Ang II regulates the expression of adhesion molecules, CD62L on human neutrophils, through AT1R. This suggest role of RAS in migration of leukocytes, during the acute inflammation, from the blood to sites of inflammation, and attachment of this cells to the endothelium at sites of inflammation[65]. Also, it has been described that the CD62L density on lymphocytes and monocytes/macrophages was significantly lower in hypertensive patients than in normotensives[66,67]. Ang II has been detected also in peripheral tissues (such as aortic tissue), suggesting possible role of the local renin-angiotensin system in atherosclerosis[68]. Both local and circulating Ang II exerts their activities through the binding to AT1R or AT2R. AT1R is widely expressed on different cell types involved in atherosclerogenesis[69]. AT2R are ubiquitously expressed in fetus and dramatically fall in the first few hours after birth[70], except in some pathological states possibly associated with inflammation[71,72].

The major RAS components, angiotensin-converting enzyme (ACE), AT1R and ATR2 were investigated on genetic level, mainly in cardiovascular phenotypes. The ACE gene has functional insertion/deletion (I/D) polymorphism of a 287 bp Alu sequence within intron 16[73]. The ACE DD carriers have higher local levels of ACE and that genotype may have a more pronounced effect due to higher Ang II on atherosclerosis and inflammatory processes in human[73-77]. A silent polymorphism (+1166 A/C) in the human AT1R gene has been associated with cardiovascular disease, possibly as a result of enhanced AT1R activity[78]. Because this polymorphism occurs in the 3'-untranslated region of the human AT1R gene, the biological importance of this mutation has always been questionable. The AT1R and miR-155 are co-expressed and that miR-155 translationally represses the expression of AT1R in vivo[79]. The C1166 allele is more frequent in atherosclerotic phenotype[78,80-82]. The molecular mechanisms underlying the regulation of AT2R expression are still waiting to be completely resolved. The human AT2R gene consists of two short noncoding exons, two introns and exon 3, which contains the complete protein coding region[83]. The AT2R transcripts with exon composition 1/2/3 and 1/3 have been observed in human heart and human failing heart[84,85]. It is not clear if intronic A/G polymorphism in AT2R at position −1332 /+1675 [86] might affect alternative splicing of human AT2R gene[87], or not (7288,). It also mediates proliferation, neointimal formation, wound healing and collagen synthesis[89-92] and affects inflammatory infiltration[71,91,93]. The expression of AT2R mRNA in human epithelial cells during inflammation in respect to genotypes was established for the first time[72].

Besides the well known important role of RAS molecules and genes in atherosclerosis[75,76,82,94,95] RAS mediates inflammation, participates in T-cell stimulation by certain antigenic peptides and influences the permeability of the blood–brain barrier[96]. Macrophages and microglial

cells play a central role in the pathogenesis of MS mediating phagocytosis and destruction of myelin. The angiotensin-converting enzyme (ACE) is released from macrophages during inflammation. The myelin fragments released during inflammatory demyelization could be potential mechanism of ACE induction in MS[96]. The AT1R is expressed on T cells and macrophages, and Ang II induces a Th1 shift via stimulation of the ATR1[97].

Detection of high levels of expression of AT1R in CNS-resident cells (astrocytes, microglia, and neurons) in EAE was demonstrated and a new role of AT1R during CNS neuroinflammation was suggested[98]. Previously, same group and others had shown that AT1R is also expressed by infiltrating macrophages and epithelial cells, and it is highly upregulated in the murine and human CNS during neuroinflammation[99,100]. AT1R is expressed on both T cells and antigen presenting cells (APC)[101]. We investigated both, AT1R and AT2R gene polymorphisms in MS patients from Serbia and found the association of AT2R polymorphism -1332A/G, located on X chromosome, with susceptibility to MS in females[102].

Matrix metaloproteinases (MMPs)

We previously mentioned the endogenous proteases, matrix metaloproteinases (MMPs), which plays very important role in inflammatory disease. Remodeling of the extracellular matrix (ECM) and cell surface by matrix metalloproteinases (MMPs) is an important function of monocytes and macrophages, the main inflammatory cells. Classical macrophage activation selectively up-regulates several MMPs in vitro and in vivo and down-regulates tissue inhibitor of MMPs-3 (TIMP-3), whereas alternative activation up-regulates a distinct group of MMPs and TIMP-3[103]. The polymorphisms in genes that code for MMPs have been investigated during last ten years in many different diseases. As the most important up to date were considered MMP-1, -2, -3, -9. Predominantly chosen for the association studies were promoter polymorphisms, which afects the promoter activity and influence gene expression. The -1607G/2G in MMP-1[104,105], 5A/6A in MMP-3[106], -1562C/T in MMP-9[107] were the most investigated ones. Our group investigated these polymorphism in carotid atherosclerosis. We found the –1607 G/2G polymorphism solely and in specific haplotypes of three analyzed MMP-1 promoter polymorphisms were significantly associated with occurrence of carotid plaques in Serbian population. Moreover, all of the observed associations were independent of other significant risk factors[108]. Also, the genotypes with 6A allele of 5A/6A polymorphism in MMP-3 gene had significantly higher susceptibility to carotid atherosclerosis[109]. The MMP-9 -1562C/T polymorphism was not associated with carotid atherosclerosis[110]. The results, which are in acordance with other findings in this filds imlicate that the lower function MMP allele is significantly associated with atherosclerosis. The new data sugest that in case of some polymorphisms, like 5A/6A in MMP-3 the allele scpecfic regulation of MMP-3 gene by transcription factor NFkappaB exist[111]. It is a promising finding toward the use of SNPs in prediction of processes in human diseases, as we know the wide and important actions of NFkappaB in inflammation.

Among first studies of association of MMPs gene polymorphisms in MS were studies performed in patients with MS from Serbia. We found significant association with susceptibil-

ity to MS for MMP-9 -1562C/T polymorphism in females[112], and association of MMP-3 6A/6A genotype, the same allele which was the risk factor for advanced atherosclerosis, with disease severity[113]. The conceptual value of these studies is the fact that some important polymorphism, e.g. 5A/6A was never studied in GWAS studies. This, similarly to ApoE or ACE gene polymorphisms, was not present on the commercial microarray SNP chips, which were mainly used in these studies. The reason is that some polymorphisms are the insertion/deletion or genotype contains more than one SNP. Thankfully to existence of some of the free access data bases we can perform that kind of check-up. The T1D base is one of those, primarily focused on the genetics and genomics of type 1 diabetes susceptibility (T1D), but enriched with the results of GWAS-es in other inflammatory diseases such as MS and celiac disease[114].

Inhibition of inflammation

Inhibitors of ACE (ACEI) and antagonists of the AT1R (ARB) are widely used drugs, predominantly in the treatment of hypertension, myocardial infarction, and stroke. The pharmacological blockade of the RAS has positive influence on clinical outcomes of patients with cardiovascular diseases independently of the blood pressure lowering effect.
It exerts potent antiatherosclerotic effects, not only through the antihypertensive pathway but also through anti-inflammatory, antiproliferative, and antioxidant properties. These drugs have further been shown to reduce end-organ damage in the heart, kidneys and brain. Blocking of Ang II production with ACE inhibitors or inhibition of Ang II signaling with AT1R blockers suppressed autoreactive TH1 and TH17 cells[99]. Besides in immune cells, Ang II was identified as a paracrine mediator, sustaining inflammation in the CNS in the EAE mouse model of MS via TGF-β. Aplication of AT1R antagonists, frequently prescribed in treatment of atherosclerosis may be useful to interrupt this proinflammatory, CNS-specific pathway in individuals with MS[98]. Molecular mechanisms underlying the protective effects of ARBs on the postischemic brain could, also, include the suppression of inflammation[115,116].

Gene expression (mRNA) and gene regulation (miRNA) in chronic inflammatory human disease

Circulating monocytes of human blood can be divided into at least two major populations according to their surface expression of CD14 and CD16. The CD14++CD16- monocytes (amounting to 80% of blood monocytes) described as 'classical' and macrophagelike CD14+CD16++ monocytes (10% of monocytes 'non-classical',). These monocyte subsets differ in their chemokine and receptor expression and features. According to that two distinct mechanisms of recruitment and functions could exist in atherosclerosis and inflammation[117-120]. Investigated mRNA revealed different expression profiles. Approximately 270 genes in humans and 550 genes in mice were differentially expressed between two subsets of monocytes[121]. Other differences included a prominent peroxisome proliferator-activated receptor gamma (PPARgamma) signature in mouse monocytes, which is absent in humans, and strikingly opposed patterns

of receptors involved in uptake of apoptotic cells and other phagocytic cargo between human and mouse monocyte subsets[121]. In MS patient population high heterogeneity was observed in terms of disease course and treatment response. Transcriptional profile of mRNA generated from peripheral blood mononuclear cells was investigated and two subsets of MS subjects were found among untreated subjects. One of the two subsets of subjects was distinguished by higher expression of molecules involved in lymphocyte signaling pathways, and that subjects were more likely to have a new inflammatory event[122].

MicroRNAs (miRNAs) are a family of highly conserved, small noncoding RNAs that post-transcriptionally repress gene expression via degradation or translational inhibition of their target mRNAs. Functionally, an individual miRNA is important as a transcription factor because it is able to regulate the expression of its multiple target genes. In addition, other genes may also be regulated indirectly by miRNAs. It is therefore not surprising that miRNAs could be the pivotal regulators in nearly all physiological and pathological processes[123,124]. MicroRNAs (miRNAs) have recently been discovered to be regulatory modulators of gene expression[125]. MiRNAs bind to the 3′ UTR of target mRNA through base pairing, resulting in target mRNA cleavage or translation inhibition[123]. On average each miRNA regulates about 200 genes, and the outcome of regulation is cell state and type specific[126]. It is therefore not surprising that miRNAs could be the pivotal regulators in nearly all physiological and pathological processes[123,124]. Their dysregulation has been associated with many diseases. Molecular analysis of plaques, lipid accumulation and inflammation in atherosclerosis can be performed at different levels such as for instance proteomics, lipidomics, or RNA analysis[127,128]. With respect to RNA analysis, the discovery and use of miRNAs may yield new diagnostic and even therapeutic options. Recent extensive studies have demonstrated that different miRNAs are highly expressed in vascular wall, and their expression or function are dysregulated in diseased vessels. Thus, miRNAs are found to play important roles in atherosclerosis initiation and progression via regulating key vascular cellular events through their target genes[129].

MS plaques are more complicated for analysis, as they are in brain, so more comprehensive analyses were driven in circulating blood cells. The expression pattern of miRNAs in circulating human classical and non-classical monocytes has been investigated recently[130] Moreover, by comparing miRNA expression in atherosclerotic plaques to healthy arteries several miRNAs have been found to be aberrantly expressed, with the majority of miRNAs displaying a two-fold or higher increase in plaques and only few miRNAs being decreased. Several miRNAs were found in atherosclerotic plaques but not in healthy vessels or either monocyte subset. The finding of low expression of miR-miR-155 (identified in non-classical monocytes) in blood monocytes was observed in this and other study[131], which is in accordance with our unpublished findings in carotid atherosclerosis and MS (unpublished results). The transcriptome analysis of currently known miRNAs, using microarray technology, was done in peripheral blood samples of treatment naive MS patients and controls. They found that in all MS subtypes miR-17 and miR-20a, were significantly under-expressed[132]. MiR-155, a typical

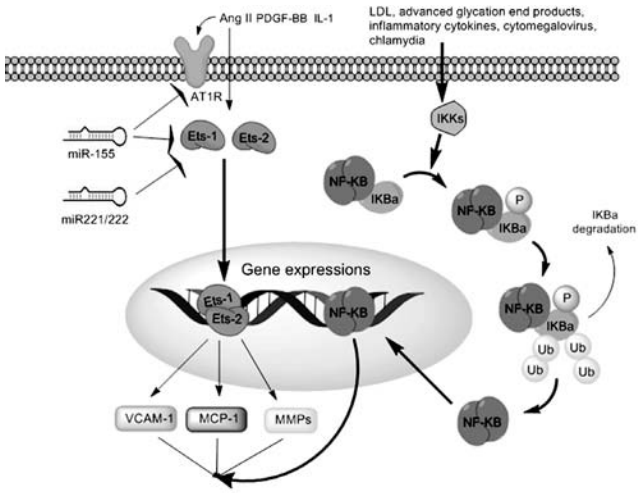


Figure 2. Summary of miRNAs implicated in endothelial inflammation upon stimulation with AngII. Inflammatory stimuli can activate the IjB/NF-jB signaling pathway, which regulates the expression of E-selectin, ICAM-1, VCAM-1, MCP-1, MMPs and P-selectin. MiR-155 can regulate endothelial inflammation by co-targeting Ets-1 and AT1. Ets-1 was also identified as a target of miR-221/222. Inflammatory stimuli induce transcription factor Ets-1 expression, which in turn activates transcription of downstream pro-inflammatory mediators VCAM-1, MCP-1 and MMPs.

multifunctional miRNA, has been found to be involved in numerous biological processes including haematopoiesis, inflammation and immunity[133]. In fact, one of the earliest described miR-155 targets is AT1R[79,134]. It was shown that AT1R and miR-155 were co-expressed in ECs and VSMCs, and miR-155 translationally repressed the expression of AT1R in vivo. A silent polymorphism (-1166 A/C) in the human AT1R had long been associated with cardiovascular disease, probably mediated by enhanced AT1R activity. Interestingly, the presence of the -1166 C-allele disrupted a miR-155 target seed region, impeding miR-155-mediated down-modulation of AT1R expression, thus allowing for increased pathological bioactivity of Ang II[79]. Our unpublished results in inflammatory core of carotid atherosclerotic plaque are in line with these findings. The recent study identify that endothelial miR-155 and miR-221/222 regulate endothelial inflammation and migration in response to inflammatory stimulation by Ang II[135]. Also there was evidence of circulating level of miR-155 that was significantly down-regulated in patients with coronary artery disease compared with healthy controls[136]. These data suggest that miR-155 and miR-221/222 play a protective role as a molecular safeguard against inflammation in atherosclerosis. Also, another miRNAs could be regulatory in endothelial inflammation in response to inflammatory stimulation. Significant increase of miR-27 expression was observed in the serum samples of atherosclerosis patients [137]. The expression of miR-27 in sera of atherosclerosis patients was positively correlated with fontaine stages, and miR-27 with different stages of atherosclerosis tells us that they may, at least in part, reflect the progression of

atherosclerosis. These results demonstrated that the serum levels of miR-27 can serve as risk factors or diagnostic markers for atherosclerosis[137,138]. In addition, most evidence suggests that miR-27 family may be a genuine proatherogenic-gene and that it may play an important role in the regulation of angiogenesis, adipogenesis, inflammation, lipid metabolism, oxidative stress and insulin signaling. Although the function of miR-27, especially its role in atherosclerosis, has not been extensively studied, there is a good reason for us to propose the further research of miR-27 in inflammatory diseases.

Inflammation and senescence

New findings suggest the shared pattern between immunity, inflammation and senescence. GWAS have identified a host of susceptibility SNPs associated with many important human chronic diseases, including inflammatory diseases and disease associated with aging. Performed meta-analysis of human GWAS revealed that 10 SNPs were significantly enriched for susceptibility to multiple diseases, 5 of which formed two highly significant peaks of disease association. These SNPs mapped to the Major Histocompatibility (MHC) locus on 6p21 and the INK4/ARF (CDKN2a/b) tumor suppressor locus on 9p21.3. The SNPs at these gene loci are previously implicated in disease pattern of MS and atherosclerosis[139]. These loci contain genes linked to either inflammation or cellular senescence pathways, and SNPs near regulators of senescence were particularly associated with disease of aging (e.g., cancer, atherosclerosis, type 2 diabetes). This analysis suggests that germline genetic heterogeneity in the regulation of immunity and cellular senescence influences the human health span. The 9p21 locus can be considered the most robust genetic marker of coronary artery disease today and shown associations with a number of additional cardiovascular disease traits, suggesting a more general role in vascular pathology. Lack of association with common cardiovascular risk factors, such as lipids and hypertension, indicated that the 9p21 locus exerts its effect through a completely novel mechanism. The ANRIL is a newly discovered non-coding RNA lying on the strongest genetic susceptibility locus in the 9p21 chromosome region. The role of this non-coding RNA in atherosclerosis progression is still poorly understood. Different splicing variants of ANRIL might have distinct roles in cell physiology. Results of recent study suggest that ANRIL splicing variants play a role in coordinating tissue remodeling, by modulating the expression of genes involved in cell proliferation, apoptosis, extra-cellular matrix remodeling and inflammatory response to finally impact in the risk of cardiovascular disease and other pathologies[140]. Also, recently, differential expression of antisense noncoding RNA in the INK4 locus (ANRIL/CDKN2BAS) was observed suggesting that ANRIL might constitute a regulator of epigenetic modification[141] The INK4b-ARF-INK4a locus is regulated by Polycomb repressive complexes (PRCs), and its expression can be invoked by activating signals. Other epigenetic modifiers such as the histone demethylases JMJD3 and JHDM1B, the SWI/SNF chromatin remodeling complex and DNA methyltransferases regulate the locus interplaying with PRCs [142]. There is also data that the INK4/ARF locus could have distinct higher-order chromatin signature in those senescent cells[143]. SNPs within the atherosclerotic risk interval may regulate ANRIL splicing and circular ANRIL (cANRIL)

production. These results identify novel circular RNA products emanating from the ANRIL locus. They suggest that causal genetic variants at 9p21.3 regulate INK4/ARF expression and atherosclerotic risk by modulating ANRIL expression and/or structure. However, only the expression of ANRIL isoforms containing exons proximal to the INK4/ARF locus correlated with the atherosclerosis risk alleles[144].

Also, in human vascular endothelial cells, interferon-γ activation strongly affects the structure of the chromatin and the transcriptional regulation in the 9p21 locus, including STAT1-binding, long-range enhancer interactions and altered expression of neighboring genes[145]. It is known that dysregulation of interferon responses may be one of the early events that contribute to the onset of MS[146]. Upregulation of interferon responses has been noted not only in a subset of MS subjects, but also in subjects with other inflammatory diseases and may reflect a shared feature of autoimmunity. Autoimmune basis of atherosclerosis was also suggested through IFNγ and Th1-driven immune responses that are also detrimental[147-149].

These findings establish a link between genetic susceptibility to various diseases and the response to inflammatory signaling, thus demonstrate the utility of genome-wide association study findings in directing studies to novel genomic loci and biological processes important for disease etiology.

Conclusion and Perspectives

The interesting is that today become obvious that intersection between atherosclerosis, most common of all of the human chronic inflammatory diseases and autoimmune inflammatory chronic diseases like MS, may go far beyond the root ''sclerosis'', which is shared in both their names. This should be the guidelines for further research.

Tissue damage evokes an inflammatory response that promotes the removal of harmful stimuli, tissue repair, and protective behaviors to prevent further damage and promote healing. However, inflammation may outlive its usefulness and become chronic. Chronic inflammation can lead to a host of diseases. So research of genes coding for some molecules with dual function in chronic inflammation, such as TRPA1, could be promising in prevention and treatment of the diseases. TRPA1 has dual function as a detector and instigator of inflammatory agents which makes it gatekeeper of chronic inflammatory disorders[150]. Also, some epigenetic modification caused by environmental stimuli could result in changes of gene stability or in gene expression which is also component of genetic basis of inflammation. Inflammation may cause aberrant DNA methylation[151]. Hodge et al. [152] have demonstrated that the inflammatory cytokine IL-6 might exert an impact on epigenetic changes in cells via regulation of a DNA methyltransferase gene. In accordance, it was demonstrated that global DNA hypermethylation was associated with inflammation and increased mortality in CKD[153]. There is also evidence of a relation between hyperhomocysteinemia and DNA hypomethylation, longitudinal studies are needed to resolve the complex interactions between aberrant DNA methylation, B vitamins, homocysteine, inflammation, and outcome in the

context of uremia. Epigenetic DNA modifications are potentially reversible by interventions with, for example, folate in man[154]. So, there is a possibility that future therapies directed at modifying the epigenome may have favorable effects on various outcomes during chronic inflammation.

Acknowledgements

This work was supported by Serbian Ministry of Education and Science Grants OI175085 and III41028.

References

1. Frohman, E.M., Racke, M.K. & Raine C.S., (2006). Multiple sclerosis—the plaque and its pathogenesis. *N Engl J Med*, **354**, 942–955.
2. Libby, P., Ridker, P.M., Maseri, A., (2002). Inflammation and atherosclerosis. *Circulation,* **105**, 1135-43.
3. Libby, P., (2002). Inflammation in atherosclerosis. *Nature* **420**, 868–874.
4. Schleser, S., Ringseis, R. & Eder, K., (2006). Conjugated linoleic acids have no effect on TNF alpha-induced adhesion molecule expression, U937 monocyte adhesion, and chemokine release in human aortic endothelial cells. *Atherosclerosis,* **186**, 337–344.
5. Chae, Y.J., *et al*., (2007). Epigallocatechin-3-O-gallate inhibits the angiotensin IIinduced adhesion molecule expression in human umbilical vein endothelial cell via inhibition of MAPK pathways. *Cell Physiol Biochem,* **20**, 859–866.
6. Van Lint, P. & Libert, C., (2007). Chemokine and cytokine processing by matrix metalloproteinases and its effect on leukocyte migration and inflammation. *J. Leukoc Biol,* **82**, 1375–81.
7. Gijbels, K., et al., (1993). Gelatinase B is present in the cerebrospinal fluid during experimental autoimmune encephalomyelitis and cleaves myelin basic protein*. J. Neurosci Res,* **36**, 432–440.
8. Chandler, S., et al., (1995). Matrix metalloproteinases degrade myelin basic protein. *Neurosci Lett,* **201**, 223–226.
9. Mun-Bryce, S. & Rosenberg, G.A., (1998). Gelatinase B modulates selective opening of the blood-brain barrier during inflammation. *Am J Physiol,* **274**, R1203–R1211.
10. Sukhova, GK., et al., (1999). Evidence for increased collagenolysis by interstitial collagenases-1 and -3 in vulnerable human atheromatous plaques. *Circulation* **99**, 2503–9.
11. Katsuda, S. & Kaji, T., (2003). Atherosclerosis and extracellular matrix. *J Atheroscler Thromb,* **10**, 267.
12. de Craen, AJ., et al., (2005). Heritability estimates of innate immunity: an extended twin study. *Genes Immun,* **6**, 167-70.
13. Saiki, R., et al., (1985). Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science,* **230**, 1350-1354.
14. Mullis, K., et al., (1986). Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. *Cold Spring Harbor Symposium in Quantitative Biology,* **51**, 263-273.
15. Lawyer, F., et al., (1993). High-level expression, purification, and enzymatic characterization of full-length Thermus aquaticus DNA polymerase and a truncated form deficient in 5' to 3' exonuclease activity. *PCR Methods and Applications,* **2**, 275-287.

16. The International HapMap Consortium.(2003). The International HapMap Project. *Nature,* **426**, 789-796.
17. The International HapMap Consortium. (2005). A haplotype map of the human genome. *Nature,* **437**, 1299–1320.
18. Rovin, BH., Lu, L. & Saxena, R., (1999). A novel polymorphism in the MCP-1 gene regulatory region that influences MCP-1 expression. *Biochem Biophys Res Commun,* **259**, 344 –348.
19. Zivkovic, M., et al., (2007). Identification of monocyte chemoattractant protein-1 gene expression in human carotid plaques. *Atherosclerosis Supplements,* **8,** PO9.
20. Tabara, Y., et al., (2003). Polymorphism of the monocyte chemoattractant protein (MCP-1) gene is associated with the plasma level of MCP-1 but not with carotid intima-media thickness. *Hypertens Res*, **26**, 677-83.
21. McDermott, DH., et al., (2005). CCL2 polymorphisms are associated with serum monocyte chemoattractant protein-1 levels and myocardial infarction in the Framingham Heart Study. *Circulation,* **112**,1113-1120.
22. Egashira, K., (2003). Molecular mechanisms mediating inflammation in vascular disease: special reference to monocyte chemoattractant protein-1. *Hypertension,* **41**, 834-841.
23. Gilbert, J., et al., (2011). MLN1202 Study Group. Effect of CC chemokine receptor 2 CCR2 blockade on serum C-reactive protein in individuals at atherosclerotic risk and with a single nucleotide polymorphism of the monocyte chemoattractant protein-1 promoter region. *Am J Cardiol*, **107**, 906-11.
24. Bazan, J.F., et al., (1997). A new class of membrane-bound chemokine with a CX3C motif. *Nature*, **385**, 640–644.
25. Imai, T., et al., (1997). Identification and molecular characterization of fractalkine receptor CX3-CR1, which mediates both leukocyte migration and adhesion. *Cell* **91**, 521–530.
26. Matloubian, M., et al., (2000) A transmembrane CXC chemokine is a ligand for HIV-coreceptor Bonzo. *Nat Immunol*, **1**, 298-304.
27. Faure, S., et al., (2000). Rapid progression to AIDS in HIV+ individuals with a structural variant of the chemokine receptor CX3CR1. *Science,* **287**, 2274–2277.
28. Lundberg, G.A., et al., (2005) Severity of coronary artery stenosis is associated with a polymorphism in the CXCL16/SR-PSOX gene. *J Intern Med*, **257**, 415-422.
29. Apostolakis, S., et al., (2009). Genetic diversity of CX3CR1 gene and coronary artery disease: new insights through a meta-analysis. *Atherosclerosis*, **207**, 8-15.
30. Veljkovic,V., et al., (2007). Application of the EIIP/ISM bioinformatics concept in development of new drugs. *Curr Med Chem*, **14**, 441-453.
31. Stojkovi}, L., et al. (2012). The association of V249I and T280M fractalkine receptor haplotypes with disease course of multiple sclerosis. *J Neuroimmunol*, **245**, 87-92.
32. Stojkovi}, L., Veljkovic, N., & Zivkovic, M., (2012). Bioinformatic prediction of functional effects of CXC ligand 16 (CXCL16) gene polymorphisms I123T and A181V. *International Conference on Bioinformatics and Computational Biology BIOCOMP BG*, Sept, Varna, Bulgaria, **p37**.
33. Stojkovi}, L., et al., (2011). The association of CXCL16 A181V and I142T gene polymorphisms with multiple sclerosis. *5th Joint triennial congress of the European and Americas Committees for Treatment and Research in Multiple Sclerosis*, Amsterdam, The Netherlands, 19.10.2011 - 22.10.2011.
34. Zivkovic, M., et al., (2011). The association of CXCL16 A181V gene polymorphism with carotid atherosclerosis in patients subjected to endaterectomy *Atheroscler Suppl*, **12**, 153-153

35. Petit, S.J., et al., (2011). The CXCL16 A181V mutation selectively inhibits monocyte adhesion to CXCR6 but is not associated with human coronary heart disease. *Arterioscler Thromb Vasc Biol,* **31**, 914-920.
36. Norman, KE., et al., (2000). P-selectin glycoprotein ligand-1 supports rolling on E- and P-selectin in vivo. *Blood,* **96**, 3585-3591.
37. Norman, KE., et al., (1995). Leukocyte rolling in vivo is mediated by P-selectin glycoprotein ligand-1. *Blood,* **86**, 4417-4421
38. Frenette, PS., et al., (2000). P-Selectin glycoprotein ligand 1 (PSGL-1) is expressed on platelets and can mediate platelet-endothelial interactions in vivo. *J Exp Med,* **191**, 1413-1422
39. Tregouet, DA., et al., (2003). SELPLG gene polymorphisms in relation to plasma SELPLG levels and coronary artery disease. *Ann Hum Genet* **67**, 504–511
40. Zivkovi}, M., et al., (2012). The sex-specific association of Met62Ile gene polymorphism in P-selectin glycoprotein ligand (PSGL-1) with carotid plaque presence: preliminary study. *Mol Biol Rep*, **39**, 6479-85.
41. Ridker, P.M., et al., (2007). Development and validation of improved algorithms for the assessment of global cardiovascular risk in women: the Reynolds Risk Score. *JAMA,* **297**, 611–619.
42. Ridker, P.M., et al., (2008). C-reactive protein and parental history improve global cardiovascular risk prediction: The Reynolds Risk Score for men. *Circulation,* **118**, 2243–2251.
43. Retterstol, L., Eikvar, L. & Berg, K., (2003). A twin study of C-Reactive Protein compared to other risk factors for coronary heart disease. *Atherosclerosis,* **169**, 279–282.
44. Pankow, J.S., et al., (2001). Familial and genetic determinants of systemic markers of inflammation: The NHLBI family heart study. *Atherosclerosis,* **154**, 681–689.
45. Dupuis, J., et al., (2005). Genome scan of systemic biomarkers of vascular inflammation in the Framingham Heart Study: evidence for susceptibility loci on 1q. *Atherosclerosis*, **182**, 307–314.
46. Fox, E.R., et al., (2008). Epidemiology, heritability, and genetic linkage of C-reactive protein in African Americans (from the Jackson Heart Study). *Am J Cardiol* **102**, 835–841.
47. Naitza, S., et al., (2012). A genomewide association scan on the levels of markers of inflammation in Sardinians reveals associations that underpin its complex regulation. *PLoS Genet*, **8**, e1002480.
48. Dehghan, A., et al., (2011). Meta-analysis of genome-wide association studies in >80 000 subjects identifies multiple loci for C-reactive protein levels. *Circulation* **123**, 731–738.
49. Okada, Y., et al., (2011). Genome-wide association study for C-reactive protein levels identified pleiotropic associations in the IL6 locus. *Hum Mol Genet,* **20**, 1224–1231.
50. Ridker, P.M., et al., (2008). Loci related to metabolic-syndrome pathways including LEPR,HNF1A, IL6R, and GCKR associate with plasma C-reactive protein: The Women's Genome Health Study. *Am J Hum Genet,* **82**, 1185–1192.
51. Reiner, AP., et al,. (2012) Genome-wide Association and Population Genetic Analysis of C-Reactive Protein in African American and Hispanic American Women. *Am J Hum Genet*, **91**, 502-12.
52. Worns, M.A., et al., (2006). Genetic and environmental contributions to plasma C-reactive protein and interleukin-6 levels—a study in twins. *Genes Immun*, **7**, 600–605
53. Wannamethee, S.G., et al., (2007). Inter-relationships of interleukin-6, cardiovascular risk factors and the metabolic syndrome among older men. *J Thromb Haemost,* **5,** 1637–1643
54. D. Fishman, D., et al., (1998). The effect of novel polymorphisms in the interleukin-6 (IL-6) gene on IL-6 transcription and plasma IL-6 levels, and an association with systemic-onset juvenile chronic arthritis. *J Clin Invest,* **102**, 1369–1376

55. Spasojevi}-Dimitrijeva, B., et al., (2010). The IL-6 -174G/C polymorphism and renal scarring in children with first acute pyelonephritis. *Pediatr Nephrol*, **25**, 2099-2106.
56. Lawlor, D.A., et al., (2003). Geographical variation in cardiovascular disease, risk factors, and their control in older women: British Women's Heart and Health Study. *J Epidemiol Community Health,* **57**, 134-140
57. Marmot, M. & Brunner, E., (2005). Cohort Profile: the Whitehall II study. *Int J Epidemiol,* **34**, 251-256
58. Wadsworth, M., et al., (2006). Cohort Profile: The 1946 National Birth Cohort (MRC National Survey of Health and Development). *Int J Epidemiol,* **35**, 49-54
59. Interleukin-6 Receptor Mendelian Randomisation Analysis (IL6R MR) Consortium, Hingorani, AD. & Casas, JP., (2012). The interleukin-6 receptor as a target for prevention of coronary heart disease: a mendelian randomisation analysis. *Lancet,* **379**, 1214-24.
60. Dandona, P., et al., (2007). Angiotensin II and inflammation: the effect of angiotensin-converting enzyme inhibition and angiotensin II receptor blockade. *J Hum Hypertens,* **21**, 20–7.
61. Ross, R., (1999). Atherosclerosis—an inflammatory disease. *N Engl J Med,* **340**, 115–26.
62. Ito, H., Takemory, K. & Suzuki, T., (2001). Role of angiotensin II type 1 receptor in the leukocytes and endothelial cells of brain microvessels in the pathogenesis of hypertensive cerebral injury. *J Hypertens,* **19**, 591–7.
63. Jacoby, D.S. & Rader, D.J., (2003). Renin-angiotensin system and atherothrombotic disease: from genes to treatment. *Archives of Internal Medicine,* **163**, 1155–1164.
64. Schieffer, B., & Drexler, H., (2003). Role of 3-hydroxy-3- methylglutaryl coenzyme a reductase inhibitors, angiotensinconverting enzyme inhibitors, cyclooxygenase-2 inhibitors, and aspirin in anti-inflammatory and immunomodulatory treatment of cardiovascular diseases. *The American Journal of Cardiology,* **91**,12–18.
65. Vega, A., et al., (2010). Angiotensin II induces CD62L shedding in human neutrophils.*Atherosclerosis,* **209**, 344-51.
66. Mills, PJ., et al. (2002). Peripheral blood mononuclear cell CD62L and CD11a expression and soluble interstitial cell adhesion molecule-1 levels following infused isoproterenol in hypertension. *J Hypertens* **20**, 311–6.
67. Zapolska-Downar, D., et al., (2006). Is there an association between angiotensin-converting enzyme gene polymorphism and functional activation of monocytes and macrophages in young patients with essential hypertension? *J Hypertens,* **24**, 1565–73.
68. Hirono, Y., et al., (2007). Angiotensin II receptor type 1-mediated vascular oxidative stress and proinflammatory gene expression in aldosterone-induced hypertension: the possible role of local renin-angiotensin system. *Endocrinology,* **148**, 1688–1696.
69. Tamura, K., et al., The role of angiotensin AT1 receptor-associated protein in reninangiotensin system regulation and function. *Current Hypertension Reports*, **9**, 121–127.
70. Gendron, L., Payet, M.D. & Gallo-Payet, N., (2003). The angiotensin type 2 receptor of angiotensin II and neuronal differentiation: from observations to mechanisms. *Journal of Molecular Endocrinology,* **31**, 359–372.
71. Esteban, V., et al. (2003). Effect of simultaneous blockade of AT1 and AT2 receptors on the NFkappaB pathway and renal inflammatory response. *Kidney Int Suppl*, **86**, S33-38.
72. Stankovi}, A., et al., (2010). Expression profiling of the AT2R mRNA in affected tissue from children with CAKUT. *Clin Biochem*, **43,** 71-75.

73. Rigat, B., et al. (1990). An insertion/deletion polymorphism in the angiotensin I-converting enzyme gene accounting for half the variance of serum enzyme levels. *J Clin Invest*, **86**, 1343-1346.
74. Suehiro, T., et al. (2004). Increased amount of the angiotensin-converting enzyme (ACE) mRNA originating from the ACE allele with deletion. *Hum Genet*, **115**, 91-96.
75. Stankovi}, A., @vkovi}, M. & Alavanti}, D., (2002). Angiotensin I-converting enzyme gene polymorphism in Serbian population: a gender-specific association with hypertension, *Scand J Clin Lab Invest,* **62**, 469-76.
76. Kolakovi}, A., et al., (2012). The association of ACE I/D gene polymorphism with severe carotid atherosclerosis in patients undergoing carotid endarterectomy. *J Renin Angiotensin Aldosterone Syst,* **13**, 141-7.
77. Kosti}, M., et al. (2004). ACE and AT1 receptor gene polymorphisms and renal scarring in urinary bladder dysfunction. *Pediatr Nephrol*, **19,** 853-857.
78. Bonnardeaux, A., et al. (1994). Angiotensin II Type 1 receptor gene polymorphisms in human essential hypertension. *Hypertension*, **24**:63– 69.
79. Martin, M.M., et al. (2007). The human angiotensin II type 1 receptor +1166 A/C polymorphism attenuates microrna-155 binding. *J Biol Chem*, **282**, 24262-24269.
80. Wang, W.Y.S., Zee, R.Y.L., & Morris, B.J.(1997). Association of angiotensin II type 1 receptor gene polymorphism with essential hypertension. *Clin Genet,* **51**, 31– 34.
81. Wu, C.K., et al. (2009). A propensity score-based case-control study of renin-angiotensin system gene polymorphisms and diastolic heart failure. *Atherosclerosis*, **205**, 497-502.
82. Stankovi}, A., et al., (2003). Angiotensin II type 1 receptor gene polymorphism and essential hypertension in Serbian population, *Clin Chim Acta,* **372,** 181-5.
83. Martin, M.M, & Elton T.S. (1995). The sequence and genomic organization of the human type 2 angiotensin II receptor. *Biochem Biophys Res Commun*, **209**, 554–562.
84. Wharton, J., et al. (1998). Differential distribution of angiotensin AT2 receptors in the normal and failing human heart. *J Pharmacol Exp Ther*, **284**, 323–36.
85. Warnecke, C., et al. (1999). Analysis and functional characterization of alternatively spliced angiotensin II type 1 and 2 receptor transcripts in the human heart. *J Mol Med*, **77**, 718–27.
86. Erdmann, J., et al. (2000). Novel intronic polymorphism (+1675G/A) in the human angiotensin II subtype 2 receptor gene. *Hum Mutat*, **15**, 487.
87. Nishimura, H., et al (1999). Role of the angiotensin type 2 receptor gene in congenital anomalies of the kidney and urinary tract, CAKUT, of mice and men. *Mol Cell*, **3**, 1–10.
88. Warnecke, C., et al. (2005). Intronic ANGII. type 2 receptor gene polymorphism 1675 G/A modulates receptor protein expression but not mRNA splicing. *Am J Physiol Regul Integr Comp Physiol*, **289**, R1729–1735.
89. Janiak, P., et al. (1992). Role of the angiotensin subtype 2 receptor in neointima formation after vascular injury. *Hypertension*, **20**, 737–745.
90. Cao, Z., et al. (1999). Role of angiotensin receptor subtypes in mesenteric vascular proliferation and hypertrophy. *Hypertension*, **34**, 408-414.
91. Cao, Z., et al. (2002). Angiotensin type 2 receptor antagonism confers renal protection in a rat model of progressive renal injury. *J Am Soc Nephrol*, **13,** 1773–1787.
92. Mifune, M, et al. (2000). Angiotensin II type 2 receptors stimulate collagen synthesis in cultured vascular smooth muscle cells. *Hypertension*; **36**, 845-850.
93. Ruiz-Ortega, M., et al. (2001). Systemic infusion of angiotensin II into normal rats activates nuclear factor-kappaB and AP-1 in the kidney: role of AT(1) and AT(2) receptors. *Am J Pathol*, **158**, 1743-1756.

94. Zivkovi}, M., et al., (2007). X-linked angiotensin II type 2 receptor gene polymorphism -1332A/G in male patients with essential hypertension. *Clin Chim Acta*, **386**, 110-3.
95. Kolakovic, A., et al., (2011). Association of Angiotensin II Type 1 receptor +1166 A/C gene polymorphism with human carotid plaque vulnerability. *Atherosclerosis suplement,* **12**, 111.
96. Constantinescu, CS., et al., (2000). Murine macrophages stimulated with central and peripheral nervous system myelin or purified myelin proteins release inflammatory products. *Neurosci Lett,* **287**, 171–4.
97. Shao, J., et al., (2003). Imbalance of T-cell subsets in angiotensin II-infused hypertensive rats with kidney injury. *Hypertension,* **42**, 31–38.
98. Lanz, TV., et al., (2010). Angiotensin II sustains brain inflammation in mice via TGF-beta. *J Clin Invest*, **120**, 2782-94.
99. Platten et al. (2009). Blocking angiotensin-converting enzyme induces potent regulatory T cells and modulates TH1- and TH17-mediated autoimmunity. *Proc Natl Acad Sci U S A,* **106**, 14948–14953.
100. Stegbauer, J., et al., (2009). Role of the renin-angiotensin system in autoimmune inflammation of the central nervous system. *Proc Natl Acad Sci U S A*, **106**, 14942–14947.
101. Nahmod, KA., et al., (2003). Control of dendritic cell differentiation by angiotensin II. *FASEB J,* **17**, 491–493.
102. Kolakovi}, A., et al., (2010). Angiotensin II receptor type 2 (AT2R) -1332 A/G gene polymorphism as a risk factor for multiple sclerosis. *ECTRIMS,* **16**, S80
103. Huang, W.C., et al., (2012). Classical Macrophage Activation Up-Regulates Several Matrix Metalloproteinases through Mitogen Activated Protein Kinases and Nuclear Factor-κB. *PLoS One*, **7**, e42507.
104. Pearce, E., et al., (2005). Haplotype effect of the matrix metalloproteinase-1 gene on risk of myocardial infarction, *Circ res,* **97,**1070
105. Affara, Met al., (2011). MMP1 bimodal expression and differential response to inflammatory mediators is linked to promoter polymorphisms, BMC. *Genomics*, **12**, 43
106. Ye, S., et al., (1996). Progression of coronary atherosclerosis is associated with a common genetic variant of the human stromelysin-1 promoter which results in reduced gene expression. *J Biol Chem*, **271**, 13055-60.
107. Zhang, B., et al., (1999). Functional polymorphism in the regulatory region of gelatinase B gene in relation to severity of coronary atherosclerosis. *Circulation,* **99**, 1788-94.
108. Djuri}, T., et al., (2012). Matrix metalloproteinase-1 promoter genotypes and haplotypes are associated with carotid plaque presence. *Clin Biochem*, [Epub ahead of print]
109. Djuri}, T., et al., (2008). Association of MMP-3 5A/6A gene polymorphism with susceptibility to carotid atherosclerosis. *Clin Biochem*, **41**, 1326-9.
110. Kolakovi}, A., (2009). Matrix metalloproteinase-9 gene polymorphism -1562 C/T as susceptibility factor for carotid atherosclerotic plaque development. *IV Congress of the Serbian Genetic Society*, June 1st-5th, Tara, Serbia, **p.90**
111. Souslova, V., et al., (2010). Allele-specific regulation of matrix metalloproteinase-3 gene by transcription factor NFkappaB. *PLoS One*, **5**, e9902.
112. Zivkovi}, M., et al., (2007). Matrix metalloproteinase-9 -1562 C/T gene polymorphism in Serbian patients with multiple sclerosis. *J Neuroimmunol*, **189**, 147-50.
113. Djuri}, T., et al., (2008) Association of the MMP-3 5A/6A gene polymorphism with multiple sclerosis in patients from Serbia. *J Neurol Sci*, **267**, 62-5.

114. http://www.t1dbase.org/
115. Ando, H., et al., (2004). Angiotensin II AT1 receptor blockade reverses pathological hypertrophy and inflammation in brain microvessels of spontaneously hypertensive rats. *Stroke*, **35**, 1726–1731.
116. Zhou, J., et al., (2005) Angiotensin II AT1 receptor blockade abolishes brain microvascular inflammation and heat shock protein responses in hypertensive rats. *J Cereb Blood Flow Metab* **25**, 878–886.
117. Weber, C., Zernecke, A. & Libby, P., (2008). The multifaceted contributions of leukocyte subsets to atherosclerosis: Lessons from mouse models. *Nat Rev Immunol*, **8**, 802–815.
118. Woollard, KJ. & Geissmann, F., (2010). Monocytes in atherosclerosis: Subsets and functions. *Nat Rev Cardiol*, **7**, 77–86.
119. Gautier, EL., Jakubzick, C. & Randolph, GJ., (2009). Regulation of the migration and survivalof monocyte subsets by chemokine receptors and its relevance to atherosclerosis. *Arterioscl Thromb Vasc Biol*, **29**, 1412–1418.
120. Swirski, FK., Weissleder, R. & Pittet, MJ., (2010). Heterogeneous in vivo behavior of monocyte subsets in atherosclerosis. *Arterioscl Thromb Vasc Biol*, **29**, 1424–1432.
121. Ingersoll, MA., et al., (2010). Comparison of gene expression profiles between human and mouse monocyte subsets. *Blood*, **115**, e10–19.
122. Ottoboni, L., et al., (2012). An RNA profile identifies two subsets of multiple sclerosis patients differing in disease activity. *Sci Transl Med*, **4**, 153ra131.
123. Bartel, DP., (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell*, **116**, 281–297.
124. Filipowicz, W., Bhattacharyya, SN. & Sonenberg, N., (2008). Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? *Nat Rev Genet*, **9**, 102–114.
125. Baltimore, D., et al., (2008). MicroRNAs: new regulators of immune cell development and function. *Nat Immunol*, **9**, 839–845.
126. Sempere, LF., et al., (2004). Expression profiling of mammalian microRNAs uncovers a subset of brain-expressed microRNAs with possible roles in murine and human neuronal differentiation. *Genome Biol*, **5**, R13.
127. Djuri}, T., et al., (2011). Association of MMP-8 promoter gene polymorphisms with carotid atherosclerosis: preliminary study. *Atherosclerosis*, **219**, 673-678.
128. Kori}anac,. G, et al., (2012). Estradiol enhances effects of fructose rich diet on cardiac fatty acid transporter CD36 and triglycerides accumulation. *Eur J Pharmacol*, doi: 10.1016/j.ejphar.2012.08.007.
129. Weber, C., Schober, A.& Zernecke, A., (2010) MicroRNAs in arterial remodelling, inflammation and atherosclerosis. *Curr Drug Targets*, **11**, 950–6.
130. Bidzhekov, K., et al., (2012). MicroRNA expression signatures and parallels between monocyte subsets and atherosclerotic plaque in humans. *Thromb Haemost*, **107**, 619-25.
131. Merkerova, M., Belickova, M. & Bruchova, H., (2008). Differential expression of micrornas in hematopoietic cell lineages. *Eur J Haematol*, **81**, 304–310.
132. Cox, MB., et al., (2010). ANZgene Multiple Sclerosis Genetics Consortium. MicroRNAs miR-17 and miR-20a inhibit T cell activation genes and are under-expressed in MS whole blood. *PLoS One*, 5e12132.
133. Faraoni, I., et al., (2009). miR-155 gene: a typical multifunctional microRNA. *Biochim Biophys Acta*, **1792**, 497–505.

134. Martin, MM., et al., (2006). MicroRNA-155 regulates human angiotensin II type 1 receptor expression in fibroblasts. *J Biol Chem*, **281**, 18277–18284.
135. Zhu, N., et al., (2011). Endothelial enriched microRNAs regulate angiotensin II-induced endothelial inflammation and migration. *Atherosclerosis* **215**, 286-293.
136. Fichtlscherer, S., et al., (2010). Circulating microRNAs in patients with coronary artery disease. *Circ Res*, **107**, 677–684.
137. Li, T., et al., (2011). Identification of miR-130a, miR-27b and miR-210 as serum biomarkers for atherosclerosis obliterans. *Clin Chim Acta*, **412**, 66–70.
138. Staszel, T., et al., (2011). Role of microRNAs in endothelial cell pathophysiology. *Pol Arch Med Wewn,* **121**,361–7.
139. Jeck, WR., Siebold, AP. & Sharpless, NE., (2012). Review: a meta-analysis of GWAS and age-associated diseases. *Aging Cell*, **11**, 727-31.
140. Congrains, A., et al., (2012). CVD-associated non-coding RNA, ANRIL, modulates expression of atherogenic pathways in VSMC. *Biochem Biophys Res Commun*, **419**, 612-6.
141. Holdt, LM. & Teupser, D., (2012). Recent studies of the human chromosome 9p21 locus, which is associated with atherosclerosis in human populations. *Arterioscler Thromb Vasc Biol*, **32**,196-206.
142. Popov, N. & Gil, J., (2010). Epigenetic regulation of the INK4b-ARF-INK4a locus: in sickness and in health. *Epigenetics*, **5**, 685-690.
143. Hirosue, A., et al., (2012). Quantitative assessment of higher-order chromatin structure of the INK4/ARF locus in human senescent cells. *Aging Cell*, **11**, 553-6.
144. Burd, CE., et al., (2010). Expression of linear and novel circular forms of an INK4/ARF-associated non-coding RNA correlates with atherosclerosis risk. *PLoS Genet*, **26**, 1001233.
145. Harismendy, O. et al., (2011). 9p21 DNA variants associated with coronary artery disease impair interferon-γ signaling response. *Nature*, **10**, 264-268.
146. De Jager, PL., (2009). Meta-analysis of genome scans and replication identify CD6, IRF8 and TNFRSF1A as new multiple sclerosis susceptibility loci. *Nat Genet*, **41**, 776-782.
147. Hansson, GK. & Hermansson, A., (2011). The immune system in atherosclerosis. *Nature Immunol*,**12**, 204–212.
148. Buono, C., et al., (2005). T-bet deficiency reduces atherosclerosis and alters plaque antigen-specific immune responses. *Proc Natl Acad Sci USA*, **102**, 1596–1601.
149. Kleemann, R., Zadelaar, S. & Kooistra, T., (2008). Cytokines and atherosclerosis: a comprehensive review of studies in mice. *Cardiovasc Res*, **79**, 360–376.
150. Bautista, DM., Pellegrino, M. & Tsunozaki, M., (2012). TRPA1: A Gatekeeper for Inflammation. *Annu Rev Physiol*, online,DOI: 10.1146/annurev-physiol-030212-183811.
151. Teitell, M. & Richardson, B. (2003). DNA methylation in the immune system. *Clin Immunol*, **109**, 2–5.
152. Hodge, DR., et al., (2001). Interleukin-6 regulation of the human DNA metyltransferase (HD-NMT) gene in human erythroleukemia cells. *J Biol Chem*, **276**, 39508–39511.
153. Stenvinkel, P., et al., (2007). Impact of inflammation on epigenetic DNA methylation: A novel risk factor for cardiovascular disease? *J Int Med*, **261**, 488–499.
154. Ingrosso, D., et al., (2003). Folate treatment and unbalanced methylation and changes of allelic expression induced by hyperhomocysteinemia in patienst with uremia. *Lancet*, **361**, 1693–1699.

CIP - Каталогизација у публикацији
Народна библиотека Србије, Београд

577.1(082)

SRPSKO biohemijsko društvo (Beograd).
Konferencija (2 ; 2012 ; Beograd)
[Proceedings] / Serbian Biochemical
Society Second Conference “Molecular
Bioscience”, 9.11.2012. Belgrade, Serbia. ;
[edited by Mihajlo B. Spasić]. - Belgrade :
Faculty of Chemistry : Serbian Biochemical
Society, 2012 (Belgrade : Colorgrafx). - 133
str. : graf. prikazi ; 24 cm

Tiraž 500. - Bibliografija uz svaki rad.

ISBN 978-86-7220-048-5 (FOC)

a) Биохемија - Зборници
COBISS.SR-ID 194357516

Supported by
Faculty of Chemistry, University of Belgrade