Serbian Biochemical Society
Fourth Conference

University of Belgrade, Faculty of Chemistry.
14.11.2014. Belgrade, Serbia.

" Biochemistry - molecular life science "

PROGRAMME

10.00-10.05	Welcome messages and information about SBS and FEBS upcoming events: Prof. M. B. Spasic (President of the Serbian Biochemical Society)
10.05-10.25	Natalija Polovic, PhD Department of Biochemistry, Faculty of Chemistry, University of Belgrade, Belgrade, Serbia **Functional roles of BPIF proteins expressed at mucosal sites**
10.25-10.45	Lidija Israel-Zivkovic, PhD Department of Chemistry, School of Medicine, University of Belgrade, Serbia **Enzymes in unusual environments**
10.45-11.05	Andjelka Petri, PhD Department of Biology ,Faculty of Science University of Novi Sad, Serbia **Molecular Evolution of the calcium binding domain of TRPP2: functional insights**
11.05-11.15	Short break
11.15-11.35	Milena Curcic, PhD Department of Biology and Ecology, Faculty of Science University of Kragujevac, Serbia **The molecular mechanism of apoptosis, redox status and synergistic effects of Allium flavum L. extracts and new-synthesized Pd(II) complex on colon cancer cells**

11.35-11.55 Zorana Orescanin –Dusic, PhD

Department of Physiology, Institute for Biological Research Sinisa Stankovic, University of Belgrade, Serbia

Ibogaine-pharmacological aspects of its action

12.30-12.50 Ivana Stojanovic, PhD

Department of Biochemistry, Medical Faculty, University of Nis, Serbia

Molecular mechanisms of CNS response to inflammation-results of experimental and clinical studies

12.50-13.10 Ivana Stojanovic, PhD

Department of Immunology, Institute for Biological Research Sinisa Stankovic, University of Belgrade, Serbia

MIF – a novel modulator of insulin activity

13.10-13.30 Svetlana Paskas, PhD

Department for Endocrinology and Immunoradiology, Institute for the Application of Nuclear Energy–INEP, University of Belgrade, Serbia

Molecular characterization of thyroid gland tumors

13.30- 14.00 Discussion and concluding remarks

Foreword

Dear Colleagues,

It gives me a great pleasure to wish you a warm welcome to the 4th Conference of the Serbian Biochemical Society entitled "Biochemistry – molecular life science"

Official language of the 4th Conference of the Serbian Biochemical Society will be English. We have invited eight lecturers from Serbia to present their achievements in their respective fields of work and their presentations will be published in the Proceedings. At the same time, we would like to use this opportunity to invite researchers from broad area of molecular life sciences for further co-operation.

Please find enclosed information on 40th FEBS Congress- The Biochemical basis of life – to be held on July, 4-9, 2015, in Berlin, Germany as well as FEBS 3+ Meeting-Molecules of life – to be held on September 16-19, 2015, Portoroz, Slovenia.

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

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

Proceedings

Functional roles of BPIF proteins expressed at mucosal sites

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Introduction

Infectious diseases had repeatedly decimated the European population in the past and had occurred as a consequence of unsanitary living conditions and lack of adequate treatment (plague, Black Death, cholera, Spanish fever, tuberculosis, anthrax). At the beginning of the third millennium, same diseases are on the increase. Human illnesses associated with bacterial infections are still one of the leading causes of death. Due to an increased incidence of antibiotic-resistant bacteria it is anticipated that this trend will continue and intensify over the coming years [1].

Antibacterial defense at mucosal sites

Upper respiratory tract, including nasal and oral cavities, is a major route of entry of pathogens into the body, and early recognition of bacterial products in this region is critical for host defense. Most common bacterial pathogen motifs associated with severe clinical effects (e.g. sepsis; inflammation) is lipid constituents of cell walls: lipopolysaccharide (LPS) of Gram-negative bacteria; lipoteichoic acids of Gram-positive bacteria and lipoarabinomannans of mycobacteria [2]. Antibacterial defense at mucosal sites rely not only on sIgA[3] and mucus secretion[4], but on expression of leukocytes and epithelial cells derived antimicrobial peptides and proteins (AMP), as well. From AMPs, defensins and cathelicidins act *via* membrane perturbation; phospholipase A2 and lysozyme act catalytically[5], while some others deprive microorganisms of important nutrients (e.g. lactoferrin)[6]. Recently, the expression of a novel family of AMPs was detected in the upper airways and nasopharynx[2]. Novel AMPs belong to bactericidal/permeability increasing protein family and are stipulated to share structural features with both neutrophils derived bactericidal/permeability increasing protein (BPI) and lipopolysaccharide binding protein (LBP)[7,8].

Bactericidal/permeability increasing protein (BPI)

BPI is a 50-kDa cationic protein of 456 residues that has been purified and cloned from the human neutrophils[9]. The crystal structure of human BPI shows a boomerang-shaped molecule formed by two similar domains (Figure 1). There are two apolar pockets on the concave surface of the boomerang; each binding a phosphatidylcholine molecule, primarily by interacting with their acyl chains; which suggests that pockets may also bind LPS acyl chains[10].

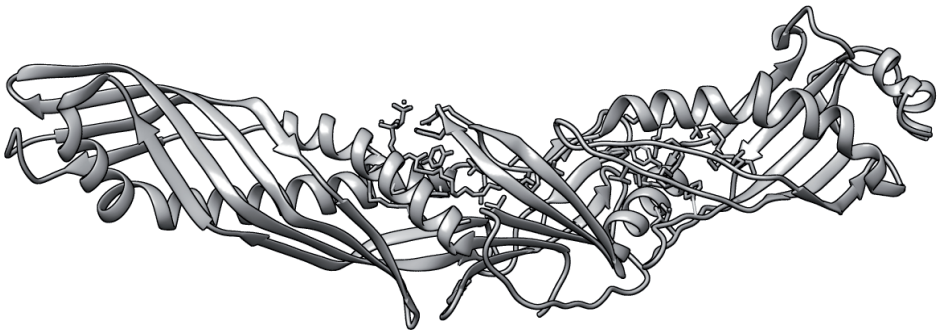

Figure 1. Structure of Bactericidal/permeability increasing protein (protein database entry 1ewf). Image was created using the program Chimera 1.8, UCSF.

In the last decade it has been extensively documented that BPI possess bactericidal, endotoxin neutralizing and opsonic activity[11]. From many known antimicrobial polypeptides, BPI stands out since at nanomolar concentrations this protein displays highly selective cytotoxicity toward a wide range of gram-negative bacterial species. Such high selectivity is a consequence of high-affinity interaction (apparent Kd ~ 5 nM) with lipid A moiety of the LPS[12]. Binding of BPI to the LPS-containing outer leaflet of gram-negative bacteria's outer membrane, competitively displaces calcium and magnesium ions which stabilize the membrane[13] leading to perturbation of the regular arrangement of LPS molecules, causing membrane rupture, and an increase in the membrane current[14]. Early effects of BPI on gram-negative bacteria include permeabilization of bacterial outer membrane rendering bacterial phospholipids susceptible to hydrolysis by phospholipases, and bacterial growth inhibition. Actual irreversible growth inhibition coincides with late effects of the bacterial inner membrane, where critical components of the bacterium's metabolic machinery reside[15].
Action of BPI against gram-negative bacteria and their endotoxins has recently been tested in animal models and in humans. Recombinant 21-kDa N-terminal BPI fragment (rBPI21) expresses both antibacterial and antiendotoxic activities[16] and has been demonstrated to have beneficial effects, either alone or in synergistic combination with conventional antibiotics, in animal models of sepsis, pneumonia, endotoxemia, and burns[11]. Moreover, rBPI21 successfully ended phase III clinical trial against severe meningococcal sepsis[17], while clinical trials for treatments of a number of infection related medical conditions are ongoing.

PLUNCs: BPI family members

The PLUNC (palate lung and nasal epithelium clone) family was introduced 35 years ago by describing parotid secretory protein expressed at rodent mucosal sites[18]. Through patterns of gene expression, conservation of genomic organization and predictions of protein structure, PLUNC proteins are related to the BPI[2]. PLUNC proteins have modest sequence similarity to related proteins, LBP and BPI. PLUNC family comprises of at least eight genes located in a single locus on chromosome 20, which are expressed in the embryonic palate, nasal epithelium and trachea[19]. PLUNC family includes proteins with structures corresponding to two-domain BPI, known as long or LPLUNC, and smaller proteins with homology corresponding only to N-terminal domain of BPI, known as short or SPLUNC. In the last decade in related literature, duplicate names for PLUNC proteins were used, but the systematic nomenclature has been recently established. According to the present nomenclature, SPLUNCn is designated as BPIFAn, while LPLUNCn is designated as BPIFBn[20].

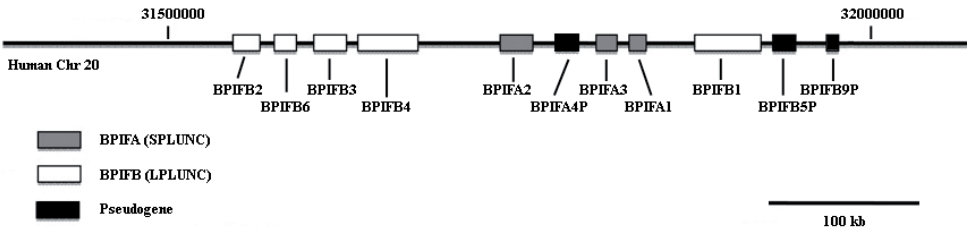

Figure 2. Genomic organization of the human PLUNC locus. The complete human PLUNC gene locus is contained within approximately 300 kb on chromosome 20q11.2.

Although multiple studies have characterized PLUNCs expression, much less has been reported regarding biochemical protein characterization, or their functional role. The human PLUNC proteins and their structure/activity reports are listed in Table 1.

Table 1. BPI horologes expressed at mucosal sites

Protein name	Expression	Protein characterization	Activity	Function
BPIFA1	Upper airways and nasopharyngs[21]	Isolated from tracheal fluid[22] X-ray structure[23]	Bacteriostatic[24] Chemoattractant[24] Regulation of sodium channel[23]	Antimicrobial[24] Mucus clearance[23] Tumor marker[25]
BPIFA2	Salivary gland tissue[2]	Isolated from saliva[26] LPS binding[26, 27]	Bactericidal[26]	Antimicrobial[26]
BPIFA3	n.a	n.a.	n.a.	Predicted: Antibacterial[28]
BPIFB1	Intestine[29] Nasal and lung epithelia[2]	LPS binding[29]	Modulation of TLR4 signaling[29]	Immunomodulation[29]
BPIFA2	Olfactory epithelium; tongue; larynx; testis; brain[30]	n.a.	n.a.	Predicted: Antimicrobial[2]
BPIFB3; BPIFB4	Olfactory epithelium[30]	n.a.	n.a.	Predicted: Odorant transport[30]
BPIFB6	n.a	n.a.	n.a.	Predicted: Immunomodulation[31]

BPIFA2 protein and *splunc2* gene presence in different species

Human BPIFA2 (SPLUNC2, parotid secretory protein – PSP) is a soluble salivary protein expressed in salivary glands and gingival epithelial cells [32, 33]. The protein has molecular weight of 25 kDa and was first identified as a product of parotid salivary glands in mice and rats[18]. Up to now, orthologous BPIFA2 proteins were found in saliva of hamster[34]; pig[35]; cattle[36] and dog[37] and BPIFA2 related genes were identified in genomes of chimpanzee; orangutan; rhesus macaque; marmoset and giant panda[38]. However, the search of genome databases identified new species with *splunc2* gene (Figure 3).

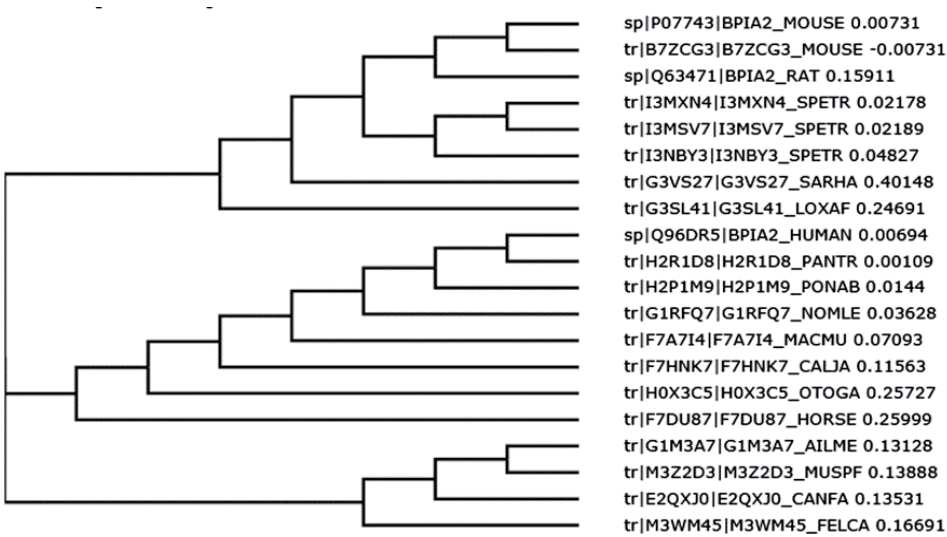

Figure 3. Evolutionary tree of BPIFA2 proteins in different species (SwissProt and TrEMBL databases search, October 2014)

Presence of BPIFA2 in different (evolutionary remote) species suggests that this protein could potentially play an important role for salivary function especially regarding its bactericidal activity.

Conclusion

The members of the PLUNC family may function in the innate immune response in regions of the mouth, nose and lungs, which are sites of significant bacterial exposure. BPI and PLUNCs are of biopharmaceutical concern as novel anti-infective agents, highlighting the potential therapeutic relevance of this protein family.

Acknowledgement

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Enzymes in unusual environments

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The idea of carrying out enzyme reaction in organic solvents was initially considered with skepticism. Now biocatalysis in organic solvents is investigated and used in numerous academic and industrial laboratories. Alkaline lipase from Pseudomonas aeruginosa NCAIM(P) B 001380, capable of growing in a water-restricted medium has excellent properties and good potential for biotechnological applications in the metal industry. Its marked stability and activity in organic solvents suggest that this lipase is highly suitable as a biotechnological tool in a water-restricted medium with a variety of applications including organosynthetic reactions and the control and prevention of Metal Working Fluid (MWF) putrification in the metal industry.

Introduction

Potential of enzymes as catalysts in water solutions is well known, but their remarkable stability and capacity to function in water-restricted environments, including organic solvents, has potentiated numerous advantages. Using organic solvent system for enzymatic reaction has importance for the bioconversion of substrates that are moderately soluble, or even insoluble, in water. The introduction of organic solvents in enzymology has provided possibility of broad variation of enzymatic reactions. The substrate, stereo-, regio- and chemoselectivities of the enzymes markedly differ in nonaqueous solutions[1]. Likewise, thermodynamic equilibrium favors synthesis over hydrolysis which has been extensively used in organosynthetic reactions. Discovery that enzymatic selectivity can be markedly affected and even inverted by the solvent is of particular importance[1]. Interactions between an enzyme molecule and the surrounding water are of the crucial importance for enzyme catalysis. The effect of organic solvents on an enzyme is primarily due to interactions with the enzyme-bound essential layer of water rather than with the enzyme itself. The water required by enzymes in nonaqueous solvents provides them sufficient conformational flexibility needed for catalysis[2]. When placed in anhydrous solvent, enzyme acquire remarkable new properties: enhanced stability, altered substrate specificity, ability to catalyze new reactions. In the absence of water, which acts as a molecular lubricant, enzymes are very rigid, so in dry solvents enzyme drive to unfold but it does not possess conformational flexibility to do so. Protein becomes kinetically ″trapped″[1].

Significant advantages of using enzymes in organic solvent systems are numerous: increased solubility of nonpolar substrates, suppression of water-dependent side reactions, thermodymnamic equilibria favors synthesis over hydrolysis, alteration of specificity, recovery and reusability of enzyme, often enhanced thermostability, and elimination of microbial contamination. Some of the disadvantages are: inactivation of enzymes, mass-transfer limitations, expensive preparation of biocatalysts, water activity control[3].

Organic solvent systems

There are three main types of organic solvent systems, depending on the miscibility with water and relative ratio of the solvent and water in the system: 1-water-water miscible organic solvent system; 2- water-water immiscible organic solvent system; 3-nearly anhydrous solvent system.

Water-water miscible organic solvent system is produced when water miscible cosolvents are added to the medium to improve the solubility of compounds. These systems are monophasic which lead to more rapid reaction rates for hydrophobic compounds by reducing the mass-transfer limitations. Moreover, this system has advantages because thermodynamic equilibrium favor synthesis over hydrolysis. The concentration of substrates and products around enzymes can be easily controlled in order to prevent an excessively high concentration of substrates or products around enzymes. However, direct contact of the organic solvent with the enzyme can result in rapid denaturation and inactivation[4].

Water-water immiscible organic solvent system consist of two phases, the aqueous containing a dissolved enzyme and the phase of an immiscible organic solvent, and between them the interfacial area forms. Inactivation rates of enzymes in two-phase systems are lower than those in cosolvents systems because the direct contact of an enzyme with organic solvent is prevented. Although minimized, denaturation of the enzyme occurs at the interface. The reaction rates are relatively low because of a low rate of mass-transfer across the interface. This obstacle can be overcome by intensive stirring. However, the increased interface can results in denaturation and inactivation of the enzyme[5]. When enzymes and substrates are soluble in the aqueous phase and products are soluble in the organic phase a water-water immiscible organic solvent system is desirable. The enzymatic reaction proceeds in the aqueous phase and product are partitioned in the organic phase, so the product yield is high and separation is easy.

Native enzymes are insoluble in nearly anhydrous solvent systems. The stability of solid enzymes is sometimes high but their activities are lower than those of solubilized enzymes. Conformation mobility of enzyme at such low water content is restricted and therefore the proteins are more rigid. The increased rigidity results in lower activity from one side but higher stability from another. Enzymes can be solubilized by modification with amphipatic compounds. In these systems conformational mobility of enzymes is restricted. The rigidity

of the enzyme structure results in higher thermal stability and provides the possibility of use of the techniques such as molecular imprinting [6]. The water participates in all noncovalent interactions maintaining the native, catalytically active enzyme conformation, so the water is indeed required for enzyme action[7]. As long as water is localized in few monolayers around the enzyme, it can be catalytically active, and this is situation depicting an enzyme functioning in nearly anhydrous organic medium[8].

Effect of organic solvents on the stability and activity of enzymes

In organic solvent containing media the protein unfolding occurs due to disturbance of the balance between hydrophobic interactions, electrostatic interactions, Van der Waals forces, and hydrogen bonds. Water plays an important role in enzyme structure and function in aqueous media. That role becomes even more important when one focuses on enzymes in low water media.

Different approaches (experiments as well as molecular dynamic simulations of enzymes) have contributed the understanding of the factors affecting stability and activity of enzymes in organic solvents. Enzyme activity in organic solvents depends on parameters such as: water activity, partition coefficient log*P*, nature of solvent, pH control, and enzyme form[3]. These parameters are often helpful in describing the behavior of enzymes in organic solvents but so far none of them have provided serious predictive analysis[9].

Analysis focused on enzyme structure, flexibility, and the details of enzyme hydration in organic solvents have been performed[10]. The enzyme surface and the active site region are well hydrated in aqueous medium, whereas with increasing polarity of the organic solvent the hydration water is stripped from the enzyme surface. Water stripping is accompanied by the penetration of organic solvent molecules into crevices on the enzyme surface and especially into the active site. More polar organic solvents replace mobile and weakly bound water molecules in the active site. In contrast, the lack of water stripping in octane allows efficient hydration of the active site. These differences in the active site hydration are consistent with the inverse dependence of enzymatic activity on organic solvent polarity and indicate that the behavior of hydration water on the enzyme surface and in the active site is an important determinant of biological function especially in low water media. Wet lab and in silico experiments show the inverse dependence of enzyme activity on the polarity of the organic medium employed[10,11].

The protonation state of the various groups of enzyme is undoubtedly important for enzyme activity. Protonation in water is controlled by pH adjustment, but this is not the same in organic solvent. A way to control enzyme protonation was developed by Zaks and Klibanov. They have showed that enzyme activity in organic solvent was dependent on pH of the solution from which the enzyme was lyophilized or precipitated. The enzyme lyophilized at its pH optimum exhibit high activity (phenomenon of pH memory) [2].

Organic solvent-tolerant lipases

Many enzymes are easily denatured and inactivated in the presence of organic solvents. Therefore protein engineering and several methods such as immobilization, modification and entrapment for stabilizing enzymes have been developed [12, 13]. But, if enzymes are naturally stable and active in the presence of organic solvents, such treatment is not necessary.

Lipases are among the most promising and important biocatalysts for carrying out reactions in both aqueous and nonaqueous media. This is primarily due to their ability to utilize a broad spectrum of substrates[14] having high chemo-, regio- and enantioselectivities in the synthesis or hydrolysis of lipid compounds, as well as their stability under a wide range of conditions. Most of organic solvent tolerant lipases have been isolated from microorganisms including organic solvent tolerant bacteria such as *Pseudomonas spp*. Their stability in organic solvents has been reported for lipases from *Pseudomonas pseudomalei* 12sm (*P. pseudomalei)*[15], *P. aeruginosa* YS-7[16], *P. Mendoncina*[17], *P. aeruginosa* LST-03[4], *P. aeruginosa* ATCC 27853[18] and *P. Fluorescens*[19]. In addition, lipases from *P. aeruginosa*, *P. cepacia* and *P. fluorescens* have been manufactured and extensively used in organosynthetic reactions, particularly in chiral production of racemic compounds[20], which includes manipulation in nonaqueous solutions. One selected organic solvent tolerant lipase is summarized below.

A lipase from *P.aeruginosa* NCAIM (P) B 001380[21]

P. aeruginosa NCAIM(P) B 001380 (previously named *P. aeruginosa* san-ai) has been isolated from putrid mineral cutting oil used as metalworking fluid (MWF) in the metal industry[22]. It was assumed that *P. aeruginosa* san-ai grown in a water-restricted medium, could secrete enzymes stable and active in organic solvents. It has been shown that the bacteria beside lipase[21] produces protase stable in organic solvents [22], biosurfactant rhamnolipid and polysaccharide[23,24]. Both enzymes, lipase and protease have shown stability and activity in organic solvents and their use as detergent additives has been evaluated[25].

Characterization of lipase protein

Basic characteristics of the lipase are presented in Table 1. The relative molecular mass of the san-ai lipase was estimated to be 54 kDa which is higher than that of other lipases from *P. aeruginosa*. The optimum pH and temperature were 11 and 70 ^{o}C, respectively. The enzyme is stabile over a broad pH range (pH 4-11.5). Remaining activities of the enzyme at 70 ^{o}C and 60 ^{o}C after 15 min were 30% and 75% of the initial activity, respectively. The lipase was inhibited strongly by Zn^{2+}, Hg^{2+}, Cu^{2+} and slightly by Ca^{2+} and Mg^{2+}. Non-ionic detergents and sodiumdeoxycholate enhanced lipase activity.The divalent metal-chelating agents EDTA and *o*-phenathroline as well as the S-S reducing agent DTT and SH-carboxymethylation reagent IAA caused no inhibition of lipase activity. The lipase preferably acted on triacylglycerols with medium-chain fatty acids.

Table 1. Characteristics of the lipase

Characteristic	
Mw	54 kDa
pH optimum	11
Temperature optimum	70 °C
Temperature stability	60 °C 15 min (75%)
Inhibition (%)	Hg^{2+}
Substrate specificity	The highest activity on middle higher faty acids

Organic solvent stability

The effects of various organic solvents: methanol, ethanol, acetone, butanol, *iso*-propanol, chloroform, *n*-hexane and DMFA, on the lipase from *P. aeruginosa* san-ai were examined at 30 °C for 2 days. The san-ai lipase was stable for 24 h in selected organic solvents, with the exceptions of *n*-butanol and *iso*-propanol. Nonmiscible organic solvents with a much higher log P, such as chloroform (2.0) and *n*-hexane (3.5) (compared with the following alcohols butanol, 0.80; *iso*-propanol, 0.28) had a stabilizing effect on the enzymatic activity in aqueous solution. The lipase retained complete activity in chloroform and *n*-hexane, even after a long exposure of 2 days. Lipase exhibit high stability in organic solvents as expected since obtained from microorganisms growing in specific medium.

Thermal stability in organic solvents

The thermal instability of enzymes is a consequence of protein unfolding on exposure to high temperature; however an improved thermal stability of enzymes in non-aqueous media has been documented[1]. The thermal stability of the lipase from *P. aeruginosa* san-ai was measured as remaining activity of the crude enzyme solution supplemented with n-hexane, DMFA, ethyl-methyl ketone, methanol and ethanol at a final concentration of 30% at 50 °C and 60 °C (Table 2.). It was found that the enzyme retained 100% activity in *n*-hexane for 15 min at 60 °C and 50% activity at the same temperature for 30 min. The enzyme retained 100% activity in methanol for 30 min at 50 °C and 75% activity in ethyl-methyl ketone for 10 min at 50 °C. However, the enzyme was not stable at 60 °C in the presence of DMFA, ethyl-methyl ketone, methanol and ethanol. The enzyme is clearly stable in organic solvents mixtures at 50 °C. Furthermore, the thermal stability in *n*-hexane was slightly improved in comparison with that in aqueous solutions.

Table 2. Thermal stability in organic solvents

Organic solvent	Remaining activity (%)	
	50°C	60°C
Methanol	100 (for 30 min)	-
Ethanol	55 (for 10 min)	-
Ethyl-methyl ketone	75 (for 10 min)	-
DMFA	100 (for 30 min)	-
n-Hexane	85 (for 30 min)	100 (for 15 min) 50 (for 30 min)

Activity in organic solvents

Although some enzymes are stable in organic solvents, their activity is reduced. To assess the potential of water to abate organic solvent-related activity loss, the san-ai lipase was tested for its activity against *p*-NPP in organic solvents supplemented with 5% water. Results have indicated that the enzyme is active against *p*-NPP in ethanol, methanol, ethyl-methyl ketone, acetone and *n*-hexane supplemented with 5% water (Table 3.). The activity in ethanol and methanol was significantly higher than that in other solvents. Alcohols are solvents capable of forming multiple hydrogen bonds, mimicking the effect of water and, thereby, enhancing enzyme action[1]. This activation effect is particularly obvious when methanol is the solvent.

Table 3. Lipase activity on *p*-nitrophenyl palmitate in 95% organic solvents

Organic solvent	Activity (mU/mg of protein)
Ethanol	5.22
Methanol	231.02
Ethyl-methyl ketone	0.12
Acetone	0.06
n-Hexane	0.13

Potential application in metal industry

During growth in MWF, *P. aeruginosa* san-ai causes the degradation of the mineral oil, a serious problem in the metal industry. In addition, the strains of *P. aeruginosa* found in MWFs have shown to possess natural resistance to many commonly used antimicrobial agents and biocides[26].

The present study describes the production, purification, and characterization of the lipase from *P. aeruginosa* san-ai grown in MWF. The high enzymatic stability of the lipase in an alkaline water-restricted medium opens the possibility for the potential application of this lipase in the construction of an intelligent Drug Delivery System (DDS) designed for the prevention of putrefication of MWF as depicted in Fig.1. In short, if the bacterium produces an extracellular lipase, then antibacterial reagents (combinations of a bacteriolytic enzyme and a sterilizer) immobilized in a water/oil/water (W/O/W) emulsion, can be released through lipase activity. The released bacteriolytic enzyme and sterilizer will then act against the bacteria- cause of the MWF putrefication, triggering its death, that way any further secretion of the lipase will be discontinued. On the other hand, this action will also stop the release of now unnecessary antibacterial agents. Thus, the construction of such a DDS could be considered as a specific enzymatically controlled on-off mechanism of antibacterial reagent delivery in the control of MWF putrefication.

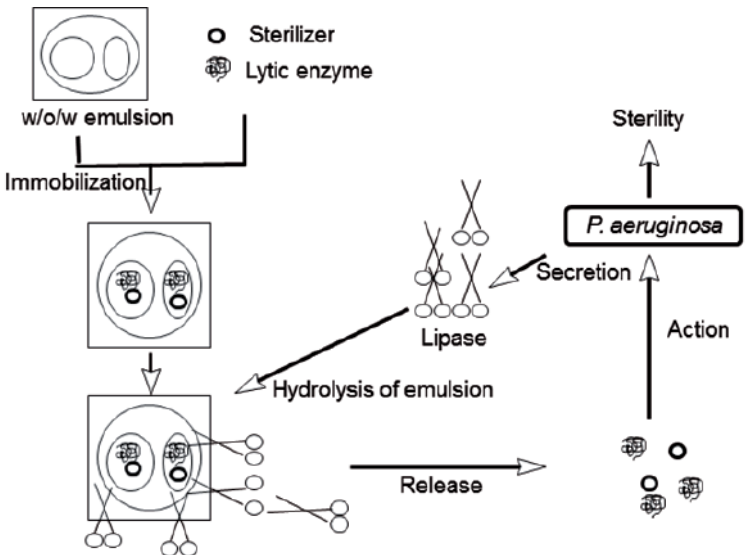

Figure 1. Model of intelligent DDS. Antibacterial agents (combinations of a bacteriolytic enzyme and a sterilizer) immobilized in a w/o/w emulsion are released by an extracellular lipase.

Enzyme immobilization

Current demand of the world's biotechnological industries is further immprovment of enzyme characteristics. Potential of different immobilisation techniques has been studied in order to overcome instability, enhance enzyme reuse, offer easier separation, making production economically profitable. Immobilization of *Candida rugosa* lipase, one representative lipase was successfully performed onto nano and meso porous materials with improvement of thermal stability and excellent reuse potential[27]. In light of the obtained encouraging results immobilisation of *P. aeruginosa* lipase and protease is underway.

Conclusion

Enzyme activity in organic solvents described as an unusual phenomenon is important instrument used im diferent aplication. Besides, it has stimulated fundamental research in order to answer questions related to the performances of enzyme in unusual environments.

Acknowledgments

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Molecular Evolution of the calcium binding domain of TRPP2: functional insights

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TRPP2 is a calcium (Ca^{2+}) permeable TRP channel whose ion channel activity is dependent on intracellular Ca^{2+} levels. Inheritable mutations in TRPP2 are associated with polycystic kidney disease (PKD) and alterations in calcium (Ca^{2+}) signaling. To better understand the relationship between TRPP2 gating and PKD associated mutations, a homology model of TRPP2 was constructed based on the cryoEM structure of TRPV1. The homology model explains known PKD associated mutations such as D511V, which cause a loss of channel activity and can be used to study channel oligomerization. These results are coupled with our continued studies of the role of the C-terminal cytoplasmic Ca^{2+} binding domain in TRPP2 channel gating. Although human TRPP2 contains two EF hand motifs, only the second is functional, *i.e.* capable of binding Ca^{2+}. Using NMR and phylogenetic analysis we show that this Ca^{2+} sensing domain has evolved, and that both EF hand motifs are capable of binding Ca^{2+} in evolutionary orthologs of TRPP2 in invertebrates. If the EF hand is responsible for the Ca^{2+}-dependence to TRPP2 channel activity; changing its' Ca^{2+} binding properties would be expected to affect the Ca^{2+} dependence of TRPP2 channels. Here we show that modified human TRPP2 channels with two Ca^{2+} binding EF hands have altered Ca^{2+} dependence. Specifically, these modified TRPP2 channels have enhanced Ca^{2+} transients in live cell fluorescent Ca^{2+} imaging, and increased ion channel activity in single channel planar lipid bilayers. The concentration of Ca^{2+} required for activating these channels appears to be lower, and the range of Ca^{2+} in which TRPP2 is active is narrower. Point mutations which disrupt Ca^{2+} binding in the second EF hand cause a decrease in Ca^{2+} transients in live cells. Together, these results strongly suggest that the EF hand region is directly responsible for sensing the concentration of Ca^{2+} required for TRPP2 channel activity, and defining the range of Ca^{2+} concentrations at which TRPP2 channels are active.

Introduction

Virtually every cell is regulated by extracellular stimuli that are transmitted to its interior. The transient receptor potential (TRP) channels form a large class of cationic channels that function as cellular sensors, allowing cells to respond to changes in fluid flow, tactile, taste, thermal, visual and osmolar stimuli in their local environment. They play a central role in sensory biology, and they have been found in all species from yeast to humans. TRP

channels are divided into seven families by distinguishing features in their cytoplasmic domains: TRPC-canonical, TRPA-ankyrin, TRPM-melastatin, TRPP-polycystin, TRPV-vaniloid, TRPML-mucolipin and TRPN-nompC.

Topology analysis predicts that all TRP channels contain six transmembrane helices (TM) with a membrane re-entrant pore loop positioned between TM5 and TM6, and cytoplasmic N- and C- terminal tails containing various functional domains. This overall topology is similar to the pore forming subunits of other 6TM channels, such as calcium-activated and voltage-gated potassium channels. Thus, although no high-resolution X-ray crystal structures of any TRP channel have been solved, their similarities with potassium channels, coupled with cryoEM structures of several TRP channels (TRPV1, TRPV4) reveal a common feature of fourfold rotational symmetry about the channel pore, consistent with a tetrameric subunit arrangement. This fourfold symmetry has also been shown by atomic force microscopy and other methods; allowing three-dimensional models of TRP channel pores to be constructed (Figure 1).

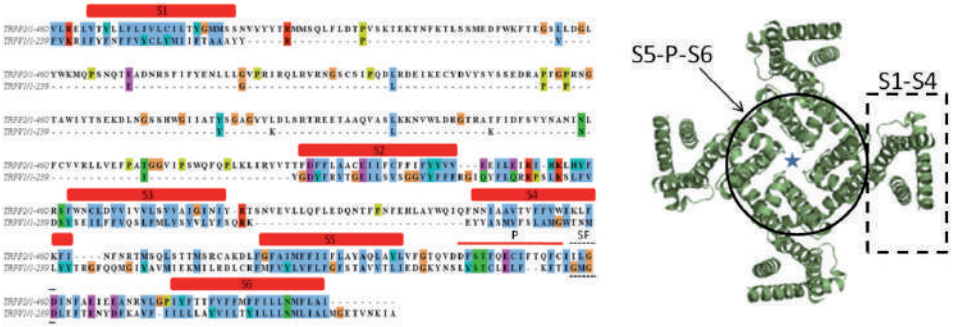

Figure 1a. Sequence alignment of the transmembrane region of TRPP2 with TRPV1. TM-transmembrane helix, P-channel pore, SF-selectivity filter. Homology model of human TRPP2 based on the cryoEM structure of TRPV1 (Bojan Krtenić, master thesis, University of Novi Sad and Krtenić et al., in preparation).

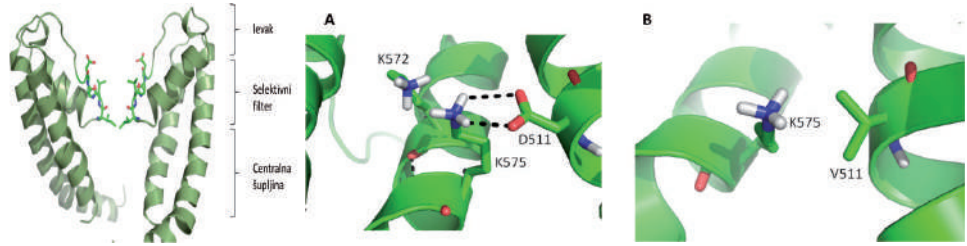

Figure 1b. Homology modeling of human TRPP2 allows visualization of the pore and selectivity filter (left), and explains the structural basis of PKD associated mutations such as D511V, which result in loss of channel activity (A and B).

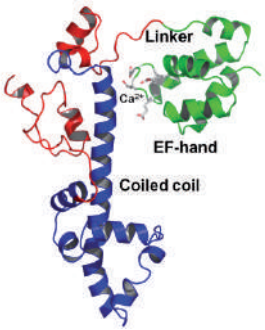

Figure 2. Structure of the C-terminal tail of TRPP2

As a class, TRP channels share little sequence similarity within and across families, except for the region encompassing TM5-TM6, the presumed ion permeation core. Differences probably stem from the need to sense very different stimuli, making it challenging to search for corresponding functional motifs in the channel polypeptide. One of the hallmarks of TRP channels is their ability to integrate multiple physiological inputs, and gating in response to these inputs is crucial. A significant amount of research has been conducted to understand how individual TRP channels, belonging to individual families detect their cognate internal and external stimuli and how they respond. However, very little is known about the molecular mechanisms governing TRP channel gating and a central issue, especially for polymodal ion channels, is to explain how different stimuli reach the same channel gate. The polymodal nature of TRP channel activation poses the following questions: 1) how do TRPs manage to sense stimuli of such diverse nature? 2) how do these stimuli translate into channel opening? and 3) how do TRPs integrate all of the input signals they receive?

We hypothesize that in spite of all of their differences, TRP channels share a common gating mechanism. This hypothesis is supported by data published over the last several years, which demonstrate that gain-of-function and loss-of-function mutations in many different TRP channels are located in the same short stretch of amino acids within TM5. Previous studies have also shown that TRP channels can form heterotetramers, strongly suggesting that each subunit undergoes similar intermolecular motions during activation.

TRPP2 is a 6-TM Ca^{2+}-permeable TRP channel that functions as an intracellular Ca^{2+}-activated Ca^{2+}-channel at the endoplasmic reticulum (ER). Conductance experiments using organic cations of increasing diameter indicate that the TRPP2 channel pore is >11Å. Assuming TM packing as in K^+ channels, at least 8 TM helices are required to form the TRPP2 pore, consistent with tetrameric assembly. In addition to independent channel functions, TRPP2 interacts with PC1 (an 11TM membrane protein) at primary cilia, where they may function as a mechanosensory channel or an effector of mechanosensory channels. Inherited mutations in TRPP2 lead to polycystic kidney disease. We have shown that the C-terminal cytoplasmic tail of TRPP2 contains several functional domains: an EF-hand domain connected by a flexible acidic linker to a coiled coil domain (Figure 2).

We hypothesize that these domains play functional roles in Ca^{2+}-dependent channel activation. TRPP2 channels are activated at low levels of Ca^{2+}, reach a maximum with increasing concentrations and are inactivated at higher levels. Phosphorylation of TRPP2 within the C-terminal tail (at S812) raises the threshold [Ca^{2+}] required for TRPP2 activation, but Ca^{2+}-dependence for non-phosphorylatable TRPP2 mutants (S812A) remain bell-shaped. In cultured mammalian cells, intracellular cytoplasmic Ca^{2+} levels are regulated by TRPP2; an effect abrogated in R742X (a missense mutation resulting in deletion of

the entire C-terminal cytoplasmic tail). TRPP2 channels are polymodal, and in addition to changes in Ca^{2+} levels, TRPP2 channel activity can be altered by interactions with other proteins. We have shown previously by Small-Angle X-ray Scattering (SAXS), NMR and Analytical Ultracentrifugation (AUC) that Ca^{2+}-binding by the TRPP2 EF-hand domain induces conformational and oligomerization state transitions in the C-terminal cytoplasmic region. Our data suggests that the coiled coil is involved in channel oligomerization and that the EF-hand is involved in Ca^{2+}-induced channel activation and inhibition.

Most ADPKD-associated mutations in TRPP2 result in truncated protein products, many of which lack the Ca^{2+} binding EF hand (*e.g.* N720X, ΔL736-N737, R742X, and Y762X; see http://pkdb.mayo.edu). The EF hand is a well-known Ca^{2+} binding motif with a common helix-loop-helix. In so-called "canonical" EF hands, a loop containing twelve amino acids, six of which coordinate Ca^{2+}, is held between two α-helices. EF hand motifs usually occur in pairs, with closely apposed EF hand motifs frequently displaying cooperative Ca^{2+} binding. For clarity, a pair of EF hands is defined as a domain, and a single helix-loop-helix EF hand as a motif. EF hands can serve as Ca^{2+} buffering proteins (*e.g.* parvalbumin or calmodulin) or Ca^{2+}-sensors, where Ca^{2+} binding induces conformational changes leading to changes in cellular signaling or activity. EF-hands functioning as Ca^{2+} sensors have been proposed to exist in voltage-dependent sodium channels, voltage-dependent calcium channels and two-pore channels.

Human TRPP2 contains a pair of EF hand motifs, which upon Ca^{2+} binding induce conformational changes in (at least) the C-terminal cytoplasmic region, which could be involved in ion channel gating. However, for human TRPP2, only the second EF-hand motif appears able to actually bind Ca^{2+}, due to deletion (possibly by evolutionary loss) of four critical amino acids in the first motif that are necessary for Ca^{2+} binding. Interestingly, sequence and phylogenetic analysis suggests that these four amino acids are absent in TRPP2 in all mammalian species, but are present in TRPP2 orthologs in evolutionarily earlier invertebrate organisms. For example, sea urchin TRPP2 (suTRPP2) contains two complete EF hand motifs, both of which are predicted to bind Ca^{2+}. The sea urchin TRPP2 ortholog is believed to play physiologically different roles (*e.g.* spermatogenesis) than human TRPP2, suggesting that evolutionary changes to the EF hand may have adapted TRPP2 channels for new functions (i.e. changes in local calcium concentrations) in mammals.

Previously we have shown that point mutations disabling the Ca^{2+} binding site in the EF hand result in loss of TRPP2 channel activity. Based on these and other studies, it is tempting to speculate that the EF hand could directly sense intracellular Ca^{2+} and delineate the Ca^{2+} dependence of TRPP2 channel activity. Using comparative structural biology and protein NMR, we found that suTRPP2 has two EF hand motifs that both bind Ca^{2+}. We used these structural results as a guide to create modified TRPP2 channels with different Ca^{2+} binding properties. Human TRPP2 channels with altered EF hand motifs were compared with wildtype channels using single channel recordings in planar lipid bilayers and live cell Ca^{2+} imaging. Our studies provide the first direct evidence that the EF hand domain determines the threshold of Ca^{2+} necessary for TRPP2 channel activation, and defines the window of Ca^{2+} in which TRPP2 channels are active.

Structural analysis of the evolution of the calcium binding domain of TRPP2 by NMR

Comparative structural biology was used to provide insight into the function of human TRPP2, through analysis of evolutionary changes. To estimate the evolutionary relationships of TRPP2 orthologs to human TRPP2, a phylogenetic tree was constructed comparing the C-terminal regions of TRPP2 proteins. Interestingly, all TRPP2 orthologs contain the same overall domain architecture in their C-terminal cytoplasmic region: an EF hand domain connected by an acidic linker to a coiled coil domain; suggesting conservation of these domains. Furthermore, the degree of sequence similarity across these C-terminal domains is high, with the TRPP2 EF hand domain having ~43% identity between sea urchin and human TRPP2 orthologs. Based on phylogeny, TRPP2 orthologs can be classified into two groups, depending only on the number of Ca^{2+} binding EF hand motifs present in their C-terminal regions (Figure 3).

Structure-based sequence alignments were then created for the EF hand domains from TRPP2 orthologs using the NMR structure of human TRPP2-EF (PDB ID 2KQ6) as a template, and compared with the canonical EF hand calmodulin (Figures 4 and 5). Sequence alignments were calculated in Clustal Omega using BLAST search results from the cytoplasmic tail of human TRPP2 and analyzed in the program JalView. For clarity, sequence alignments have been truncated to show only the EF hand domains. The NMR structure of human TRPP2 EF was used to model the secondary structure of TRPP2 EF hand orthologs. As can be seen, all TRPP2 orthologs appear to contain two helix-loop-helix EF hand motifs based on similarity to human calmodulin and comparison with a 'consensus' EF hand sequence. The second EF hand motif is highly conserved across all TRPP2 orthologs, and appears to be functional, with all residues necessary for Ca^{2+} binding. However, the first EF hand motif appears to be non-functional in vertebrate TRPP2 orthologs, due to the evolutionary loss of four critical residues necessary for Ca^{2+} coordination.

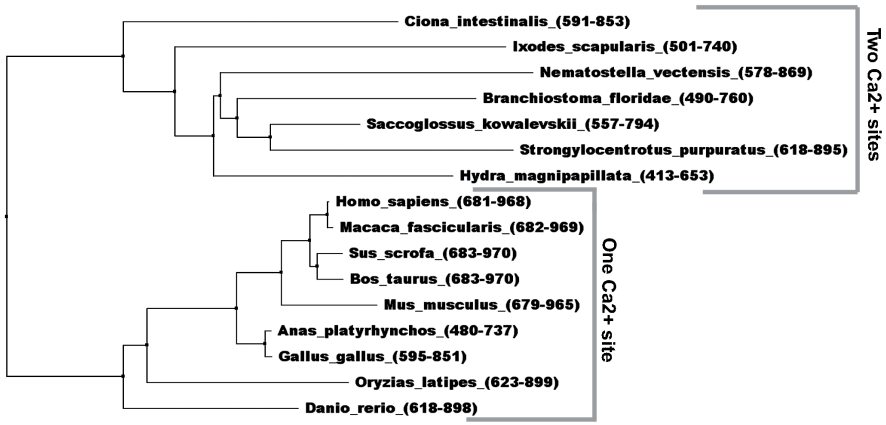

Figure 3. Phylogenetic analysis of the C-terminal cytoplasmic region of TRPP2

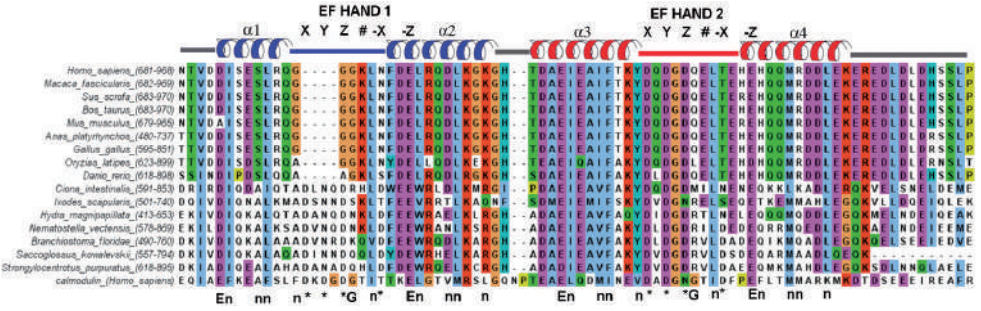

Figure 4. Structure-based sequence alignment of the EF hand domain of human TRPP2 with TRPP2 orthologs.

Figure 5. Structure-based alignment of the EF hand domain of human TRPP2 with calmodulin (a canonical EF hand)

Sea urchin TRPP2 is the most well-studied invertebrate ortholog of TRPP2. Thus, we focused on the sea urchin TRPP2 EF hand domain as representative of other orthologs. To determine if both EF hand motifs in sea urchin TRPP2 (suTRPP2) can bind Ca^{2+}, ^{15}N-HSQC NMR spectra were recorded for sea urchin TRPP2 EF hand domain (suTRPP2-EF) in saturating (20 mM) Ca^{2+} conditions. ^{15}N-HSQC NMR spectra show that suTRPP2-EF forms a well-ordered, α-helical structure typical of EF hand domains. In addition, under saturating Ca^{2+} concentrations, chemical shifts for Q675 and R709 were significantly shifted to ~10 (^{1}H) PPMs (Figure 6), a diagnostic signature of Ca^{2+} bound EF hands, where the amide proton of each residue participates in hydrogen bonding with the side-chain carboxyl oxygen atom (C′O) of the corresponding aspartate residue at the +X position of the individual EF hand motifs. Note that this shift was not observed for loop resides in the first EF hand motif of human TRPP2-EF.

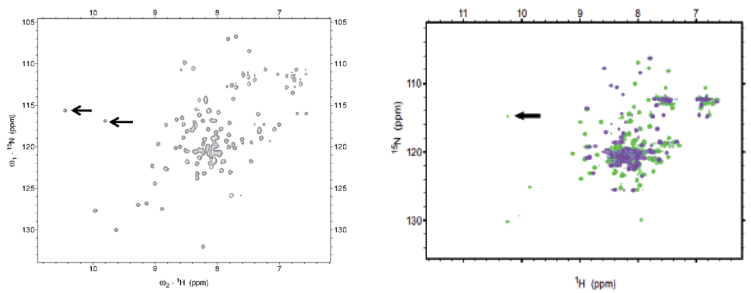

Figure 6. Chemical shifts for Q675 and R709 in the sea urchin TRPP2 EF hand domain are indicative of Ca2+ binding. Overlayed 1H-15N HSQC NMR spectra of hTRPP2-EF (dark contours) and 'DANA' hTRPP2-EF (light contours). Arrow points to amide protons involved in hydrogen bonding with the side-chain carboxyl oxygen atom of the corresponding aspartate residue in the DANA construct, indicating the presence of a second Ca2+ binding site.

Human and sea urchin TRPP2 EF hand domains have structural differences affecting Ca^{2+} binding

Because human and sea urchin TRPP2 channels are expected to play different physiological roles, and contain different numbers of functional Ca^{2+} binding sites, we propose that the TRPP2-EF domain may have evolved to respond to different levels of cytoplasmic Ca^{2+}. Thus, we compared the structures of human and sea urchin TRPP2-EF. NMR structural studies were conducted on the suTRPP2 EF hand domain (suTRPP2-EF) in saturating (20 mM) Ca^{2+} conditions. The overall fold of suTRPP2-EF is similar to other canonical EF hand domains, with two opposed helix-loop-helix EF hand motifs (residues K655 to C688, and residues D693 to L734), connected by a short linker of four amino acids (R689 to A692). However, between human and sea urchin TRPP2-EF there are key differences which may impact the Ca^{2+} dependence of TRPP2 channel activity. First, because of four missing residues in the loop of hTRPP2-EF: there is no Ca^{2+}-binding "pocket" due to a lack of key amino acids necessary to coordinate Ca^{2+}. Second, helix 1 in suTRPP2-EF is longer than in hTRPP2-EF, possibly because the presence of two Ca^{2+} binding sites in suTRPP2-EF helps stabilize helix 1. Finally, helices α1 and α2 of the first EF hand motif in suTRPP2-EF are roughly perpendicular, enabling the α1-α2 loop to assume the proper geometry for Ca^{2+} coordination, as expected for canonical Ca^{2+} binding EF-hand motifs. In contrast, α1 and α2 are parallel in human TRPP2-EF, consistent with loss of the Ca^{2+} binding site. Interestingly, interhelical angles between α3 and α4, which form the second EF hand motif, are very similar between human and sea urchin TRPP2-EF. In fact, additional NMR experiments show that point mutations disrupting the ability of site 1 to bind Ca^{2+} do not affect the structure of the second EF hand (Figure 8). This is consistent with the structure of human TRPP2 EF hand, where only site 2 can bind Ca^{2+}, but the domain remains stable. Together, these results suggest that the second EF hand motif contains a structurally stable Ca^{2+} binding site which is necessary in both vertebrate and invertebrate TRPP2 orthologs.

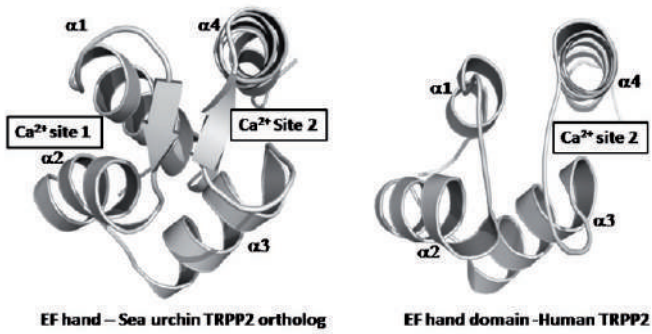

Figure 7. NMR structure of Ca^{2+} bound sea urchin TRPP2-EF (left) vs. human TRPP2-EF. Note evolutionary loss of Ca^{2+} binding site 1 in human TRPP2.

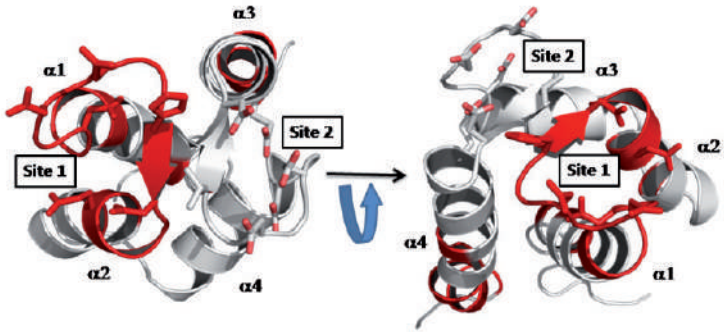

Figure 8. CA (Ca) NMR chemical shift changes observed in sea urchin TRPP2-EF with the first EF hand mutated vs. wildtype sea urchin TRPP2-EF. Chemical shift deviations > 0.5 ppm are shown in dark grey. Only EF hand 1 (in which Ca^{2+} binding –x-z residues have been mutated to alanine) experiences significant shift differences, indicating that the second EF hand, which is conserved in human TRPP2, is structurally stable (Jovana Plavša, master thesis, University of Novi Sad).

Structure-guided mutagenesis of the TRPP2 EF hand domain to create modified human TRPP2 channels with different Ca^{2+} binding potentials. Using the NMR structure of sea urchin TRPP2-EF as a guide, we created two modified human TRPP2 constructs designed to re-introduce the "evolutionarily lost" EF hand residues necessary for Ca^{2+} binding in the first EF hand motif (Figure 9). The first hTRPP2 construct, hTRPP2-"GANA" (insertion of residues 'ANAD' between D732 and G733), reintroduced four residues evolutionarily lost from hTRPP2-EF site 1. These residues coordinate Ca^{2+} in the same region of sea urchin TRPP2-EF. This modification provides two residues at the +Y and +Z position in the first EF hand of human TRPP2, enabling the site to bind Ca^{2+}. The second hTRPP2 construct, hTRPP2-"DANA" (G732D and insertion of 'ANAD' between D732 and G733), attempts to address the preference by canonical EF hands of an ASP residue at the +X position. Almost all known EF hand structures, including sea urchin TRPP2-EF, contain an aspartate residue at the +X position. Thus, the G732D substitution in hTRPP2-GANA would be expected to improve the Ca^{2+} binding potential of the TRPP2 EF hand. The ability of these modified hTRPP2-EF constructs to bind Ca^{2+} has been demonstrated by NMR (Figure 6).

```
                ____Alpha 1____      _____Alpha2____      __Alpha 3__                ___Alpha 4___
HU_PC2          KNTVDDISESLRQGG----GKLNFDELRQDLKGKGH--TDAEIEAIFTKYDQDGDQELTEHEHQQMRDDLEKEREDLDLDHSSLP
SU_PC2         KRDKIADIQEALAHADANADQHLDFDEWRQELKCRGH--ADADIEAVFAKYDVDGDRVLDAEEQMKMAHDLEGQKSDLNNQLAELE
HU_GANA         KNTVDDISESLRQGGANADGKLNFDELRQDLKGKGH--TDAEIEAIFTKYDQDGDQELTEHEHQQMRDDLEKEREDLDLDHSSLP
HU_DANA         KNTVDDISESLRQGDANADGKLNFDELRQDLKGKGH--TDAEIEAIFTKYDQDGDQELTEHEHQQMRDDLEKEREDLDLDHSSLP
HU_-X-Z         KNTVDDISESLRQGG----GKLNFDELRQDLKGKGH--TDAEIEAIFTKYDQDGDQELAEHAHQQMRDDLEKEREDLDLDHSSLP
HU_DANA_-X-Z    KNTVDDISESLRQGDANADGKLNFDELRQDLKGKGH--TDAEIEAIFTKYDQDGDQELAEHAHQQMRDDLEKEREDLDLDHSSLP
Calmodulin       EQIAEFKEAFSLFDKDGDGTITTKELGTVMRSLGQNPTEAELQDMINEVDADGNGTIDFPEFLTMMARKMKDTDSEEEIREAFR
                              X Y Z #-X -Z                          X Y Z #-X -Z
```

Figure 9. Creation of modified human TRPP2 channels with different Ca^{2+} binding properties.

Functional analysis of the evolution of the calcium binding domain of TRPP2

Re-introduction of the evolutionarily lost Ca^{2+} binding site to the EF hand domain of human TRPP2 changes the Ca^{2+} dependence of TRPP2 channel activity and alters TRPP2 dependent Ca^{2+} signaling. Single channel experiments in planar lipid bilayers were used to compare modified TRPP2 channels (hTRPP2-DANA and hTRPP2-GANA) with wildtype human TRPP2 (Figure 10). To monitor the Ca^{2+} conducting properties of TRPP2 channels, and to determine if modifications to the first EF hand motif altered the Ca^{2+} dependence of TRPP2 channel activity, we tested the single channel activities of both the hTRPP2-GANA and hTRPP2-DANA constructs vs. wildtype human TRPP2. Over the Ca^{2+} range tested, we found that both GANA and DANA hTRPP2 channel variants displayed higher open channel probabilities at the optimal Ca^{2+} concentration than wildtype hTRPP2. In addition, the concentration range over which Ca^{2+} modulates channel activity was narrower, with a shift to lower Ca^{2+} concentrations. These single channel experiments provide the first evidence that alterations to the Ca^{2+} binding properties of the TRPP2 EF hand motif directly change the Ca^{2+} dependence of TRPP2 channel activity, as well as channel open probability.

We then examined the effects of these variants on Ca^{2+} signaling in live cells (Figure 10). The amplitude of Ca^{2+} response transients from hTRPP2-GANA were significantly higher than wildtype hTRPP2. The duration of the response (time taken to return to 25% of the maximal response) for both hTRPP2-GANA and hTRPP2-DANA was significantly longer vs. vector control and wildtype hTRPP2. Similar results were recorded using Ca^{2+} free conditions, indicating that Ca^{2+} was released from intracellular stores. These results suggest that reintroduction of the evolutionarily lost Ca^{2+} binding site in the EF hand domain enhances Ca^{2+} signaling, and that the enhanced Ca^{2+} transients can be attributed to modifications to the Ca^{2+} binding properties of the TRPP2 EF hand domain. It is likely that the effect of adding the second EF hand has a larger impact on the sustained Ca^{2+} induced Ca^{2+} response, via TRPP2 and/or InsP3R. These results are in agreement with our single channel studies, and provide additional evidence supporting the hypothesis that TRPP2-EF is responsible for the Ca^{2+} dependence of TRPP2 channel activity.

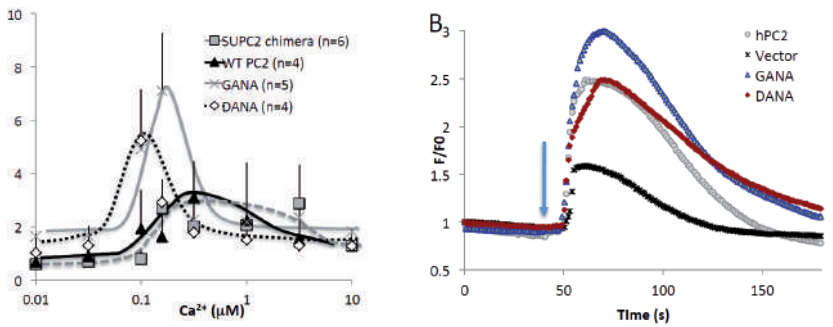

Figure 10. Single channel recordings (open probabilities). B) Live cell calcium transients.

Discussion

We propose that these EF hand motifs serve as a Ca^{2+} sensor responsible for the Ca^{2+}-dependence of TRPP2 channel activity. Using NMR and bioinformatics, we show that the overall fold of this Ca^{2+} sensor is conserved, but that both EF hands are capable of binding Ca^{2+} in evolutionarily earlier TRPP2 orthologs. If the EF hand is truly a Ca^{2+} sensor, changing its' Ca^{2+} binding properties should change the Ca^{2+} dependence of TRPP2 channel activity. Supporting this, modified human TRPP2 channels with two Ca^{2+} binding EF hands display significantly altered Ca^{2+} dependence and increased ion channel activity in single channel recordings, as well as enhanced Ca^{2+} transients in live cell Ca^{2+} imaging. Strikingly, the threshold Ca^{2+} concentration required for TRPP2 channel activation is significantly lower, and the Ca^{2+} response window is narrower. Together, these results strongly suggest that the EF hand is directly responsible for sensing the concentration of Ca^{2+} required for TRPP2 channel activity, and defining the window of Ca^{2+} in which TRPP2 channels are active.

Conclusions and future directions

The EF hand in human TRPP2/TRPP2 senses the threshold concentration of Ca^{2+} required for TRPP2 channel activation and inhibition. However, little is known about how gating is regulated within the TRPP2 channel itself: Which residues are involved? and How are signals sensed by the cytoplasmic tail translated into channel openings and closings? Our homology model of human TRPP2 could be used to study of channel homo- and hetero-oligomerization, as well as the molecular basis of channel gating within the channel pore.

Acknowledgments

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The molecular mechanism of apoptosis, redox status and synergistic effects of Allium flavum L. extracts and new-synthesized Pd(II) complex on colon cancer cells.

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Considering that induction of apoptosis is one of the main strategies in cancer therapy, the molecular mechanisms of cytotoxic effects of *Allium flavum* L. on colon cancer cell lines were investigated, applied in single and co-treatments with new-synthesized Pd(II) complex. *A. flavum* showed cytotoxic effects on colon cancer cells (IC_{50}=1.64-84 μg/ml), but not on untransformed human skin fibroblasts. Combinations of plant extract with Pd(II) complex caused synergistic effects and lower IC_{50} values. Pd(II) complex induced high percentage of necrosis in a single treatment, but in the combination with plant extracts it had better proapoptotic and lower necrotic activity. Treatments and co-treatments influenced apoptosis biomarkers, leading to Fas protein overexpression and activation of caspases 8 and 9. *A. flavum* extract induced changes in redox status in HCT-116 and SW480 cells, increased $O_2^{\cdot-}$ and caused changes in GSH level, inhibited iNOS protein expression and NO production. All the investigated parameters indicate presence of oxidative stress and pro-oxidative effect of *A.flavum* extract in colon cancer cells, which is associated with cytotoxic and apoptotic ability of the extract.
In view of the achieved anticancer properties, insufficiently investigated *A. flavum* is a promising candidate for developing new anticancer compounds and deserves further research and scientific validation. Due to its synergistic actions with the newly synthesized chemical anticancer agent and ability to reduce side effects (thus reducing necrotic activity of Pd(II) complex), *A. flavum* can be used as a supplement to chemotherapy, perhaps in combination with currently used chemotherapeutics or in some strategies in cancer therapy.

Introduction

Colon cancer is one of the most frequent causes of cancer morbidity and mortality worldwide[1]. Therefore, the importance of better understanding the role of diet in the prevention of colon cancer is necessary, as well as discovering new natural medicinal agents with proapoptotic activity, as one of the main strategies in the treatment of cancer. Plants contain a variety of bioactive compounds that may have anticancer effects, and impressive number of recently used drugs have been isolated or derived from natural sources, based on their use in traditional medicine [2,3]. In recent years there has been a great interest in testing synergistic effects and drug combinations of compounds from natural sources and chemotherapeutic drugs[4]. Drug combinations together with the use of natural dietary supplements to chemotherapy may increase the efficacy of treatment, simultaneously decreasing its side effects. Also, such combination may improve the destruction of cancer cells through apoptosis (rather than necrosis), or reduce drug resistance thus facilitating body detoxification of chemotherapeutics[5]. Induction of apoptosis is increasingly valued as a biologically significant anticancer mechanism and one of the major strategies in cancer therapy[6].

Allium species have been used in the traditional medicine for centuries[7]. The preventive actions of garlic (*A. sativum*) and other *Allium* species against colon cancer have been demonstrated in human colon cell lines[8] and animal models[9]. Epidemiological studies indicated the correlation between *Allium* vegetable consumption and a reduced risk of colorectal carcinoma[10]. There are only a few data that confirm *A. flavum* as a plant with good antioxidant[11,12] and cytotoxic effects on a few cancer cell lines [12,13]. To our knowledge there is no data concerning molecular mechanisms of cytotoxic activity of *A. flavum*. Cisplatin is commonly used chemotherapeutic compound in anticancer therapy[14]. There are numerous attempts to create new nonplatinum metal-based complexes with good solubility and at the same time capable to be stablein cell and bind to DNA. Among them, palladium(II) complexes are very interesting. The development of palladium complexes as anticancer drugs has been promising, because of their similar structure with cisplatin[15]. A new Pd(II) complex with thiohydantoin type of ligand was synthesized in an attempt to obtain a compound with higher cytotoxicity and minimum side effects[16].

The aims of the study are investigations of *A. flavum* cytotoxic and proapoptotic effects, molecular mechanism of induced apoptosis, effects on some parameters of redox status and some metabolic enzymes, as well as synergistic effects with new-synthesized Pd(II) complex.

Cytotoxity

In this study, different *A. flavum* extracts (methanol, ethylacetate and acetone) were investigated for cytotoxic activity on colon carcinoma cell lines (HCT-116 and SW480). *A. flavum* has been used traditionally as a food supplement or tea, but the data about its anticancer properties were insufficient. Treatments with *A. flavum* extracts caused significant growth inhibition of cancer cell lines, with IC_{50} values in the range of 1.64-84 μg/ml. *A. flavum* extracts were cytotoxic on SW480 cells with IC_{50} values lower than 30 μg/ml, which

was considered a good cytotoxic activity for crude extracts[17] and selectively cytotoxic on HCT-116 cells. The extracts were not cytotoxic on fibroblasts, as the normal cell line (IC_{50} values higher than 200 μg/ml; data not shown). As an example, Table 1shows *in vitro* cytotoxic activity of *A. flavum* methanol extract, determined by MTT cell viability assay[18] and expressed by IC_{50} values (concentration which inhibits 50% of cell growth), as a parameter of cytotoxity.

The results indicate that *A. flavum* extracts had a good cytotoxity on colon cancer cell lines, without effects on normal skin fibroblasts, which confirms their anticancer activity. *A. flavum* is rich in phenols, as a potentially responsible component for cytotoxity. Caffeic acid glycosides are more dominant compounds in *A. flavum* methanol extract. Caffeic acid has a variety of pharmacologically potential effects and an inhibitory effect on proliferation of cancer cells[19]. Other polyphenols contained in *A. flavum* extract also possess cytotoxic effects on cancer cell line[20]. SW480 cells showed higher sensitivity to treatments in comparison to HCT-116 cells, with lower IC_{50} values. HCT-116 cells have defects in DNA mismatch repair systems, with mutation in the hMLH1 gene[21] which is a possible cause for resistance of this type of cancer cells[22]. SW480 cells do not have defects in DNA mismatch repair systems[23] and consequently they are more sensitive to treatments in comparison to HCT-116 cells.

Table 1. Growth inhibitory effects– IC_{50} values (μg/ml) of *A. flavum* extract in single treatments and co-treatments with Pd(II) complex on HCT-116 and SW480 cell lines

Plant extract		Cell line	24 h	72 h
A. flavum		HCT-116	28.29±0.99	35.09±1.87
		SW480	22.20±1.13	20.87±1.37
A. flavum	0.1 μMPd	HCT-116	12.85±0.13	10.51±1.01
	10 μMPd		1.60±0.05	1.74±0.08
	0.1 μMPd	SW480	7.49±0.08	9.43±0.25
	10 μMPd		4.10±0.04	0.06±0.001

Several studies have shown that chemotherapeutic drugs have harmful effects on health and can lead to the development of drug resistance in tumor cells, which limits the clinical success of cancer chemotherapy[24]. The reports show that chemotherapeutic drugs and natural compounds with known anticancer activity could be used in combination in the therapy to reduce the systemic toxicity of chemotherapeutic agents[5]. For the investigation of potential synergistic cytotoxic effects, the plant extract and new synthesized potential antitumor agent - Pd(II) complex were administred simultaneously in co-treatments in HCT-116 and SW480 cells. Good cytotoxic activity of Pd(II) complex was described previously (IC_{50} values were 16.98 μM and 6.51 μM after 24 and 72 hours, respectively, for HCT-116 and 15.73 μM and 8.89 μM after 24 and 72 hours, respectively for SW480 cells)[16]. Pd(II) complex was used in very low concentrations, 0.1 μM showed no significant cytotoxic activity in single treatment, while 10 μM inhibited cell growth for approximately 30%[16]. *A. flavum* extract in co-treatment with Pd(II) complex exerted an increased growth inhibitory effect with lower

IC_{50} values (calculated in relation to the concentrations of plant extract) (Table 1). *A. flavum* extracts combined with low doses of Pd(II) complex resulted in synergistically enhanced cytotoxic activity on HCT-116 and SW480 cells.

Fluorescence microscopic analysis of cell death

Phytochemicals can interfere and modify basic functions in cancer cells (cell cycle, apoptosis, angiogenesis, invasion and metastasis)[25]. The induction of apoptosis is an important mechanism of chemoprevention and chemotherapy of cancer[6]. To determine whether the inhibition of cell proliferation by *A. flavum* extract was due to the induction of apoptosis, the acridine orange/ethidium bromide method and specification of morphological shape of cells by fluorescence microscope were done[26]. The treated cells clearly showed morphological changes such as reduction in size and cell volume, cell shrinkage, membrane blebbing, chromatin condensation, nuclear fragmentation and formation of apoptotic bodies of treated cells.

The percentage of viable, apoptotic and necrotic cells for two incubation periods with extract was noted. In comparison to spontaneous apoptosis observed in control cells, the treatment with *A. flavum* extract caused increased number of apoptotic cells in different percentage, depending on concentration, type of extracts and cell line. Generally, extracts had proapoptotic activity on both cell lines, while necrosis appeared in a small percentage. As opposed to *A. flavum* extract, Pd(II) complex with good cytotoxic activity induced very significant necrosis on both cell lines. Only the lowest concentration (0.1 μM) showed no necrotic activity.

Co-treatments of Pd(II) complex with *A. flavum* extract decreased toxicity of Pd(II) complex on untransformed human skin fibroblasts and on contrary increased cytotoxicity on cancer cells, exhibiting synergistic effects. Similarly to MTT assay, AO/EB method showed a decreased number of viable cells and an increased number of apoptotic cells in co-treatments of plant extract and Pd(II) complex, in compare to single treatments. It is important to note that 10μ MPd(II) complex, which induced high percentage of necrosis (about 25% on HCT-116 cells and 15% on SW480 cells) in co-treatments with plant extract decreased cell viability (had better proapoptotic activity), with no necrotic effects shown.

Activated apoptotic pathways

For monitored treatments and co-treatments with extraordinarily cytotoxic and proapoptotic activity, some molecular mechanisms and apoptotic pathway were examined, which is important from the view point of potential drug design. Different kinds of caspases have a crucial role in apoptotic pathway. As a response to anticancer chemotherapy, caspases can be activated through the extrinsic (death receptor mediated) or intrinsic (mitochondria mediated) apoptotic pathways[27]. The extrinsic apoptotic pathway begins with the activation of death receptors on the plasma membrane, such as Fas/CD95. Binding of FasL induces

Fas trimerization, which recruits initiator caspase 8 via the adaptor protein FADD (*Fas-associated death domain*). The activated caspase 8 stimulates apoptosis via two parallel cascades: directly cleaving and activating effector caspases, such as caspase 3[28], or cleaving Bid (*BH3 domain–containing proapoptoticBcl2 family member)*, a proapoptotic Bcl-2 family protein. Bid translocates to mitochondria, where it triggers cytochrome c release, which sequentially activates caspase 9 and 3[29].

Monitored parameters: Protein expression of Fas receptors

For the Fas receptor protein expression and localization, the cells were analyzed by immunofluorescence staining[30]. The obtained images demonstrate that both cell lines, HCT-116 and SW480, express Fas receptors (Figure 1). Fluorescence microscopy revealed that the Fas receptors were unequally present in control and treated cells. In treated HCT-116 and SW480 cells, Fas receptors were more frequent than in control cells. Fas expression in both cell lines in treatment by Pd(II) complex was abundant.

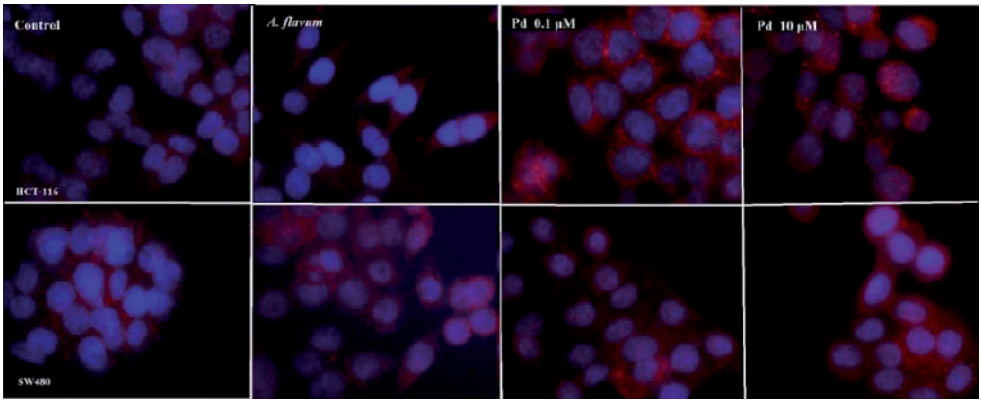

Figure 1. Fas receptors expression in HCT-116 and SW480 control cells and cells treated with 50 μg/ml *A. flavum* methanol extracts and Pd(II) complex. Cells were incubated 24 h with treatments. The images were taken using fluorescence microscopy at 600×. Nuclei were stained blue, Fas receptors were stained red.

Caspase 8 and 9 activities

The obtained results show that caspase 8 activities increased in the treated in comparison to control HCT-116 and SW480 cells (Figure 2). Caspase 8 was less active in co-treatments than in single treatments in SW480 cell line, while in HCT-116 co-treatments, it caused higher activity.

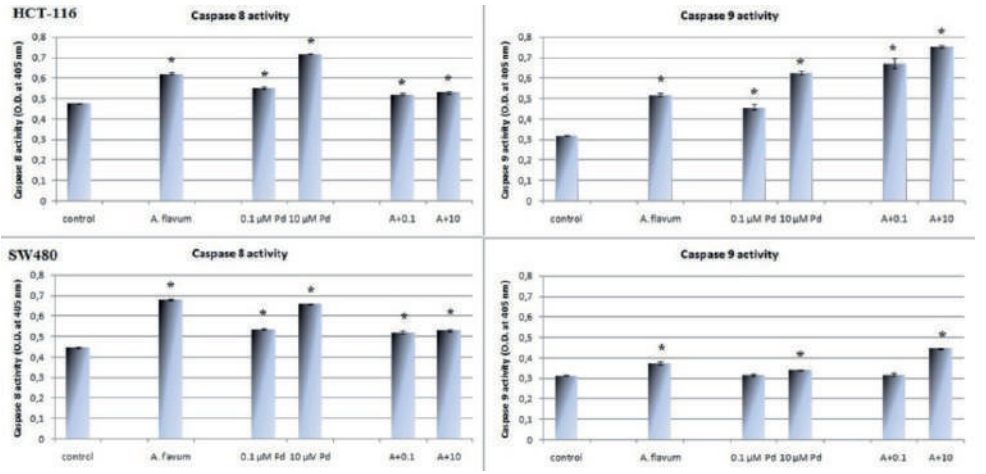

Figure 2. Caspase 8 and caspase 9 activities in HCT-116 and SW480 control cells and cells treated with 50 μg/ml *A. flavum* methanol extract, Pd(II) complex and their co-treatments. Cells were incubated 24 h with treatments. The data are mean ± SD of three independent experiments. *P < 0.05 compared to untreated controls. A+0.1 - 50 μg/ml extracts of *A. flavum* in co-treatments with 0.1 μM Pd(II) complex; A+10 - 50 μg/ml extracts of *A. flavum* in co-treatments with 10 μM Pd(II) complex.

The caspase 9 assay was performed in HCT-116 and SW480 cells in order to investigate whether the treatment with plant extracts and Pd(II) complex included mitochondria and activated intrinsic pathway of apoptosis. The caspase 9 activities increased in the treated HCT-116 and SW480 cells compared to untreated control cells (Figure 2). In the co-treatments, increased caspase 9 activities were observed in comparison to single treatments. In HCT-116 cells caspase 9 was more active than in SW480 cells.

In the monitored treatments and co-treatments, overexpression of Fas receptors and caspase 8 activation suggests that apoptosis was activated through extrinsic pathway. On the other hand, the increase in caspase 9 activity supports the fact that apoptosis induced by the treatment with plant extracts and Pd(II) complex implicated mitochondria and intrinsic apoptotic pathway. The higher activity of caspase 9 observed in HCT-116 in comparison to SW480 cells is in correlation with the literature data, which suggests that Bid cleavage is the limiting step in SW480 cells, supporting slower rate of cytochrome c release from mitochondria and caspase 9 activity in SW480 cells in comparison to HCT-116 cells[31].

In general, a distinction between HCT-116 and SW480 cells can be observed. HCT-116 cells showed higher caspase 9 activity and lower Fas protein expression in comparison toSW480. Co-treatments in SW480 cells triggered mitochondrial rather than Fas mediated pathway, preferably causing an increase in $O_2^{\cdot-}$ production and caspase 9 activation in comparison to Fas and caspase 8 activation. In HCT-116 cells, co-treatments equally activated caspases and caused a reduction of $O_2^{\cdot-}$ concentration.

Effects on redox status

Reactive oxigen (ROS) and nitrogen species (RNS), such as superoxide anions, hydrogen peroxide, nitric oxide and peroxynitrite radicals, play an important role in oxidative stress related to the pathogenesis of various important diseases like cancer, cardic reperfusion abnormalities, kidney and liver disease. Also, they have important role in cell proliferation, apoptosis and regulation of different sygnal pathway in the cells[32,33]. Production of ROS and RNS can act as initiators of apoptosis by increasing of mitochondria membrane permeability, resulted in cytochrome c relasing and inducting of apoptosis[34]. Natural antioxidants, especially from plant sources including phenolics, flavonoids, tannins, lignans and others, are widespread in food of plant origine as well in different medicinal plants. Plant phenolics may interfere with some redox sygnal pathways and function as potent free radical scavengers, reducing agents, protect against lipid peroxidation or prooxidants[35,36].

Monitored parameters: Superoxide anion concentration
Nitrobluetetrazolium assay[37] was performed to test whether the treatments by *A.flavum* methanol extract scavenged or stimulated superoxide anion ($O_2^{\cdot-}$) production.

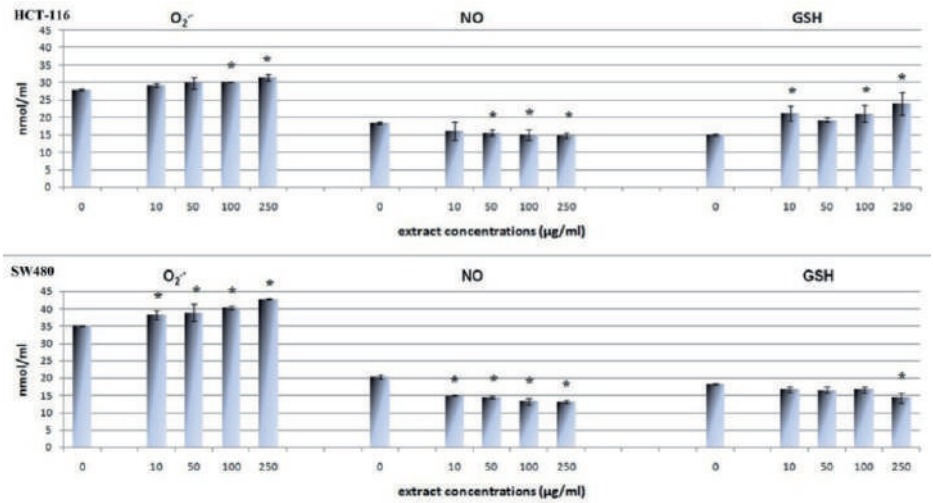

Figure 3. Effects of *A. flavum* methanol extract on $O_2^{\cdot-}$, NO (as a indicator of nitrite) and GSH concentrations, expressed as nmol/ml, in HCT-116 and SW480 cells. * statistically significant differences ($p<0.05$) compared to values in control cells.

The results of NBT assay indicate that *A. flavum* extracts induced $O_2^{\cdot-}$ production in the treated cells (Figure 3). This $O_2^{\cdot-}$ production is in correlation with cytotoxity. The cytotoxic effects and induction of apoptosis of cancer cells by some polyphenol compounds and plant extracts are partially due to their pro-oxidant actions[38]. The literature data show that

among polyphenols founded in *A. flavum* extraxt, caffeic acid glycosides and quercetin exert prooxidant properties[39,40]. They can initiate apoptosis in response to reactive oxygen species (ROS)[41], leading to increassing the expression of death receptors or changes of mitochondrial membrane permeability, relasing cytochrom c and caspase 9 activation[33].

Concentration of nitrites

Nitric oxide (NO), with important physiological functions in organism, is synthesized in biological systems by different enzymes - nitric oxide synthases[42,43]. *In vitro* data supports the ability of NO to protect human carcinoma cells from apoptosis by variety of mechanisms including enhancing the stability of the anti-apoptotic protein Bcl-2 via S-nitrosylation[44] and inhibiting the pro-apoptotic activity of caspase-3[45]. *A. flavum* extract caused inhibition of iNOS protein expression (Figure 4), directly leading to a decrease of NO concentration. Inhibition of iNOS protein expression and NO depletion enhances sensitivity to treatments by some anticancer drugs[46]. Thus, that targeted inhibition of iNOS and iNOS-derived NO may be an effective therapeutic approach for carcinoma and other iNOS-expressing tumors. Our results are coincident with other study[47,48], suggesting that some compounds in extracts (the most likely phenolic compounds) may be linked to intracellular target molecules involved in NO production pathway and resulted in inhibition of NO production by suppressing iNOS expression. Also, NO has a half-life only several seconds in environment rich in $O_2^{\cdot-}$. Superoxide anion radical has a high affinity for NO forming peroxynitrite anion ($ONOO^-$), probably reducing the concentration of NO in treated cell samples[49].

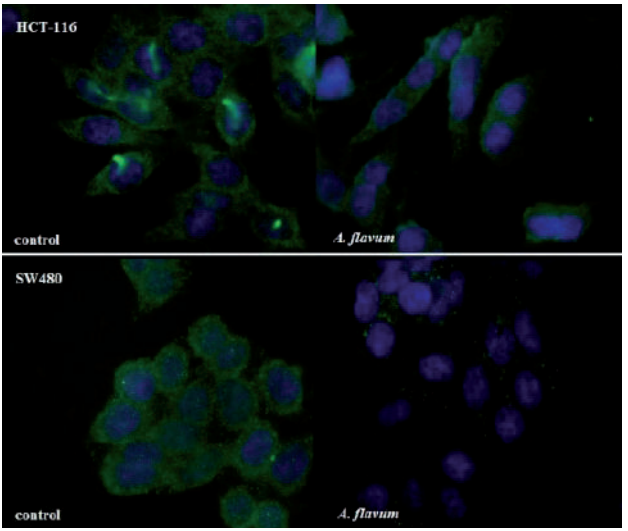

Figure 4. iNOS protein expression in HCT-116 and SW480 control cells and cells treated by 50 µg/ml of *A. flavum* methanol extract. Cells were incubated with extracts for 24 h. The images were taken using fluorescence microscopy at 600×. Nuclei were stained blue, iNOS was stained green.

Concentration of glutathione

Glutathione (GSH), the major intracellular non-protein thiol, plays an important role in a number of cellular functions, including enzyme activity, membrane transport, DNA synthesis and inactivation of xenobiotics and reactive intermediates[50]. Oxidative stress in cells generally involves the GSH system, therefore level of GSH was measured as a very important parameter of oxidative stress in control and treated cells[51].

In treated HCT-116 cells increased GSH level and antioxidant capacity in the cells was observed (Figure 3), as a response to oxidative stress. Tendency for increasing of GSH content in treated cells suggest that phenolics from extract can enhance the antioxidant status and GSH level in the cells[52] or cells de novo synthesize glutathione as consequence the production of reactive metabolites, such as $O_2^{\cdot-}$. In treated SW480 cells decreased GSH level was observed, suggesting to increased GSH exploiting in environments of high concentration $O_2^{\cdot-}$, which produce cytotoxic substances. Exploiting of GSH in the cells during the process of apoptosis is a common change[53].

Obtained results in HCT-116 and SW480 cells indicated that *A. flavum* extract increased $O_2^{\cdot-}$, caused changes in GSH level, inhibited iNOS protein expression and NO production. All the investigated parameters indicate presence of oxidative stress and pro-oxidative effect of *A. flavum* extract in colon cancer cells, which is associated with cytotoxic and apoptotic ability of the extract. SW480 cells were more sensitive on oxidative stress than HCT-116 cells. In HCT-116 cells antioxidant capacity was enhanced, with higher GSH level and lower changes in $O_2^{\cdot-}$ and NO production.

Effects on metabolic enzymes

Metabolism of xenobiotics, including anticancer drugs, occurs through several phases. Phase I is catalyzed by CYP protein family, which is involved in drug metabolism and detoxification[54]. Especially important is CYP1 classes of these enzymes and their members CYP1A1, CYP1A2 and CYP1B1[55]. Metabolism of hydrophobic compound occurs by conjugation with glutathione by enzyme glutathione S transferase (GST), in phase II. GSTP1 classis of GST enzymes is directly involved in drug resistance in many cell lines[56,57]. This conjugates may be transported out of the cell by ABC membrane transporters (phase III), such as P-gp, MRP1 and MRP2[58,59]. This enzymes and transporters play a crucial role in protecting the healthy cells from the harmful effects of carcinogens, however in cancer cells it can lead to drug resistance. Because of that possible treatment of cancer involves the invention or synthesis drugs which inhibit their activity. Targeting of these enzymes by substances from natural origin is potential success in prevention and cancer therapy[60,61].

Treatment by *A. flavum* extract inhibits iRNK expression of the investigated gene for metabolic enzymes (*CYP1A1*, *GSTP1* and *MRP2*) in the both cell lines (Figure 5). Similarly results are observed for some other plants or bioactive substances presented in them[62-65], like a quercetin, presented in *A. flavum* extract[66,67]. Phenolic compounds from plants can be substrate for these enzymes or can inhibit their activity[68]. Inhibitory activity of *A.flavum* can be useful results and suggest on ability to combined treatment of *A. flavum* extracts or their bioactive components with appropriate anticancer drugs, which is metabolized by CYP enzymes.

As opposed to plant extract, treatments by Pd(II) complex induced different effects on iRNK expression of metabolic gene. Generally, iRNK expression was unchanged or increased in treatments by Pd(II) complex, indicating to normal or increased activity and metabolism by this enzymes. Pd(II) complex increased iRNK expression of *CYP1A1* gene in SW480 cells, indicating that CYP1A1 enzyme is involved in metabolism of Pd(II) complex, as well as iRNK expression of *GSTP1* and *MRP2* gene, suggesting on possible conjugation of Pd(II) complex with GSH by GSTP1 enzyme and transport conjugates by transporter protein through cell membrane. There are no significant changes in iRNK expression of gene in HCT-116 cells.

Drug metabolism can be modified by other drugs and these interactions have great clinical importance. Interactions between natural products and anticancer drugs can be competitive, non-competitive or with no effect in terms of inhibition or induction of the enzymes responsible for their metabolism[69]. Many plants showed effects in combinations with drugs which are substrate for CYP and GSTP1 enzymes. If drug is substrate for metabolic enzymes or transporter protein, plant extract which is also substrate can displace the drug from the binder, the synergistic or antagonistic effects between the plant and the drug are based on competition for the same target site[70]. Plant or bioactive constituents can inhibit expression of transporter protein[71]. Our results demonstrate that expression of genes involved in anticancer drug metabolism, was generally decreased in co-treatment, compared to single treatment on both cell lines, which resulted in increased cytotoxicity of Pd(II) complex, because of its decreased metabolism and transport through cell membrane in the presence of plant extracts, since they inhibited expresion of these enzymes.

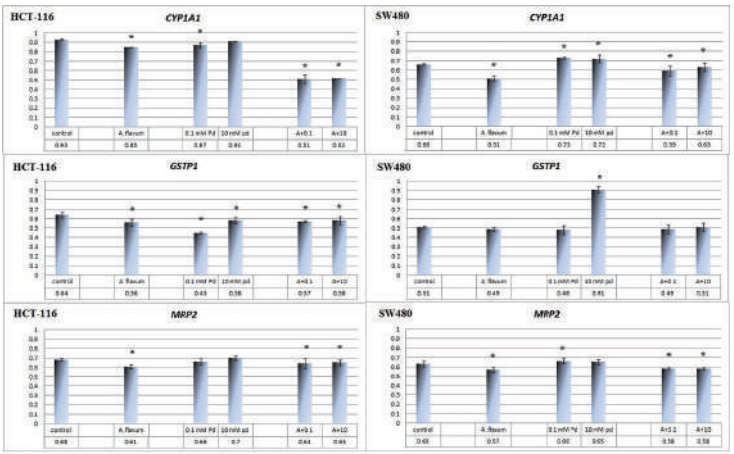

Figure 5. iRNK expression of genes (*CYP1A1*, *GSTP1* and *MRP2*) in control and treated HCT-116 and SW480 cells (values were obtained using ImageJ software and presented by columns). Relative expression was determined relative to β-actin expression in the all samples, as a positive control. *P < 0.05 compared to untreated controls. A+0.1 - 50 μg/ml extracts of *A. flavum* in co-treatments with 0.1 μM Pd(II) complex; A+10 - 50 μg/ml extracts of *A. flavum* in co-treatments with 10 μM Pd(II) complex.

Conclusion

Based on our results *A. flavum* can be considered a potential source of bioactive components with anticancer activity or be used as a dietary food supplement or supplement to chemotherapy, due to its synergistic effects with apparent chemical drugs. This study have great impact on investigation of new anticancer substances from natural source, focus on the importance of using plants as the source of medicinal drugs, contribute to the development of the appropriate therapy and give contribution in both scientific and practical means. With regards to inhibitory activity on gene expression of metabolic enzymes, it is necessary to highlight the possibility of co-treatments of tested plant extracts or their bioactive compounds with the appropriate chemotherapeutic, in order to reduce the resistance of malignant cells, as one of the greatest problems in the tumor therapy. This finding is also important from a nutritional point of view, because this extracts contain significant amounts of bioactive constituents, which provide health benefits.

Acknowledgements

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Ibogaine - pharmacological aspects of its action

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Ibogaine is a naturally occurring indole alkaloid with psychotropic and metabotropic effects obtained from the root of the *Tabernanthe iboga* plant. Ibogaine attracted widespread scientific attention due to its anti-addictive properties against cocaine, morphine, heroin, alcohol and nicotine. The pharmacological properties of ibogaine have been researched for 100 years. Pharmacology of ibogaine is quite complex because it affects many different neurotransmitter systems simultaneously. Ibogaine binds to several types of receptors: 5-Hydroxytryptamine (5-HT), opioid, nicotinic and N-methyl-D-aspartate (NMDA) receptors, dopaminergic and 5-HT transporters and monoamine oxidase enzyme (MAO).
Recent results show that ibogane has stimulating influence on rat brain energy metabolism. Experiments performed on yeast S. cerevisiae suggest that induction of energy metabolism-related enzymes is neither mediated through receptor bindings nor is it linked to cell differentiation or organization in the tissue. Our results demonstrated that ibogaine caused dose-dependent relaxation of both spontaneously and calcium-induced smooth muscle contractions of isolated rat uterus, followed by changes in antioxidative activity in the tissue. Ibogaine acts as a pro-antioxidant that increases the activities of antioxidative enzymes, as well as an adaptagene in oxidative distress.
Taken all together, these results show that some of the effects of ibogaine seem to be mediated through its influence on the energy metabolism and redox-active processes. Further explanation of ibogaine pharmacology could elucidate its diverse effects, which would allow ibogaine to return to the list of medicaments for treatment of addiction.

Introduction

Ibogaine is a naturally occurring indole alkaloid with psychotropic and metabotropic effects obtained from the root of the *Tabernanthe iboga* plant. Native tribes in Gabon have used the Iboga plant root for centuries for medicinal purposes, and in rites of passage ceremonies[11]. Ibogaine (Figure 1.) is considered to be a drug that enables the user to reach depths of the subconscious or, in smaller doses, it acts as a stimulant.

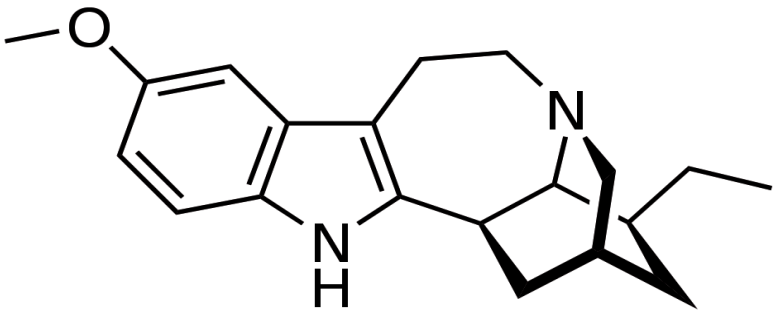

Figure 1. Ibogaine

Ibogaine attracted widespread scienific attention due to its antiaddictive properties against multiple drugs of abuse[5, 16]. Considering that preclinical and clinical developments showed the mechanism of action of ibogaine in the treatment of drug addiction that appears to be distinct from other existing pharmacotherapeutic approaches, shortage of financial supports led to stopping of further research of ibogaine in this direction. However, ibogaine is still the focus of many researches.

The pharmacological properties of ibogaine have been researched for more than 100 years. Tablets made from the extracts of the *Tabernanthe manii* root were sold in France under the trade name Lambarene to treat fatigue, depression and recovery from infectious disease[28, 17]. The next conventional preparation based on ibogaine was Iperton used as a tonic stimulant[31]. In 1985, ibogaine was patented for the use in opioid withdrawal[26]. However, pharamaceutical authorities expressed concern and criticism for its usage and during 1990s the use of ibogaine moved to alternative and informal settings. The main reason for criticism was incomplete information on its pharmacological and physiological modes of action i.e. the pharmacological and physiological effects of ibogaine were not entirely understood.

Ibogaine absorption and distribution

Animal experimentation showed that plasma levels of ibogaine after oral administration are approximately threefold higher in female than in male rats, and the bioavailability of ibogaine is approximately twofold higher in female than in male[37]. Bioavailability of ibogaine is up to about 50% in males and 75% in females. It is tissue specific and reaches micromolar concentrations[37]. After oral administration, hepatic excretion takes place. Therefore, the route of application for ibogaine is essential. Concentrations of ibogaine 1h after administration were one hundred times greater in fat and thirty times greater in brain

than in plasma. This was in accordance with its prolonged actions and lipophilic nature, which suggests that adipose tissue could be the reservoir of ibogaine[28]. Platelets or some other blood components are also proposed to be the reservoir of ibogaine, given that the concentrations were higher in the whole blood than in plasma[4]. Ibogaine is metabolized via cytochrome P-450 2D6 (CYP2D6) isoform to noribogaine. Both ibogaine and noribogaine are eliminated via renal and gastrointestinal tract with a half-life ranging from 12 to 24 hours. Noribogaine is excreted more slowly than ibogaine, and this is the reason for the persistance of its effects[27].

Pharmacology

Pharmacology of ibogaine is quite complex and affects many different neurotransmitter systems simultaneously. Ibogaine binds to several types of receptors: 5-Hydroxytryptamine (5-HT), opioid, nicotinic and N-methyl-D-aspartate (NMDA) receptors, dopaminergic and 5-HT transporters and monoamine oxidase enzyme (MAO) [4, 15, 25]. Therefore, the complexity of ibogaine's pharmacological action lies in the interaction between different levels of synaptic transmission. The effects will depend on applied dose and tissue distribution, while the most prominent effect will take place on the most sensitive receptor and effector(s). This means that the first effect will take place in brain, but the receptor effectiveness will also depend on the number of receptors. First of them are σ_2 receptors[22, 27], but not σ_1. The second are NMDA, since ibogaine is a noncompetitive NMDA antagonist[39, 24]. However, ibogaine does not induce the same effects as classic NMDA antagonist MK801[6]. On the other hand, since ibogaine is very efficient in opioid withdrawal, its action at opioids is suggested[29, 30]. Ibogaine as an agonist binds to μ-opioid receptors, but without the direct antinociceptive effects[13, 10]. It was suggested that ibogaine could enhance opioid reception indirectly down-steam of adenylate cyclase (AC), since ibogaine itself did not inhibit AC[38]. However, pharmacological studies showed that ibogaine also bend to κ-opioid receptors with significant affinity[24] that could be antagonized by κ-opioid antagonist[15, 16]. Ibogaine also binds to serotonin transporter, (1) increasing serotonin release and levels[39, 27] and (2) inhibits serotonin uptake[40]. Serotonergic transmission is also suggested to be the mechanism for ibogaine-mediated hallucinogenic effects[40]. It has been reported that ibogaine is also a weak 5-HT_{2A} agonist[19]. Ibogaine has affinity towards dopamine transporter[9], thus decreasing dopamine concentration and increasing levels of dopamine metabolites[6]. Ibogaine is a weak muscarinergic receptors inhibitor, but the evidence for the significance of this mechanism is indirect, since ibogaine-mediated cardiac dysfunction can be reversed by atropine[8]. In fact, most of the evidence regarding its pharmacological mode of action came from experiments where physiological effects were considered after treatment with ibogaine and extrapolated to possible involvement of specific type of receptors. However, many different crosslink signaling paths are involved in behavioral and physiological effects and extrapolation to individual receptors is not always simple, especially in the case of ibogaine.

Ibogaine long-lasting effects

Ibogaine itself has hallucinogenic effects and there were several attempts to distinguish the type of receptor that is involved in this state. None of them identified the exact specific receptor, despite the evidence that 5-HT_{2A} and 5-HT_{2C}, as well as σ_2, κ- and μ-opioid receptors were involved (ibogaine-mediated hallucination was only partially antagonized by their antagonist) [19, 20].
Within classical pharmacological investigation it was recently shown that ibogaine-mediated inhibition to SERT is noncompetitive[23]. However, recent results showed that ibogaine inhibited the steady state and substrate-induced currents in both SERT and DAT only from the extracellular side, increasing the accessibility of the all inward-open LeuT structures that lined the cytoplasmic permeation pathway[9, 12]. In SERT, when the cytoplasmic face of the transporter is open, cysteine residues placed at LeuT positions react with aqueous reagents in membrane fragments from cytoplasm.
On the other hand, its action on σ-2 receptors in liver requires membrane lipid rafts[14]. Preparations of receptors with Triton X-100 maintain pharmacological characteristics, decreasing its potency to bind σ-2 receptors. These suggest that ibogaine in doses applied for medical treatment could interfere with different membrane components, such as membrane ATP pools, but not through changes in membrane fluidity.

Some effects of ibogaine last much longer than a pharmacokinetic model could support. For example, mood elevating effect usually appears a day or two after application and lasts from days to weeks, when neither the substance itself nor its metabolites are still present in measurable quantities[36, 21]. Thus, it is supposed that, other than acute effects on receptor and enzyme sites, more complex biochemical, neuroendocrine and possible structural and functional changes in terms of brain plasticity also occur[3, 18]. There are data that show that after ibogaine treatment, the level of neurotensin-like immunoreactivity and substance P are increased[1, 2], as well as the secretion of corticosterone from the adrenal cortex and prolactin from the anterior pituitary[7], probably via central mechanism. However, there are still not sufficient evidence that could explain the mechanisms of persistent ibogaine effects. The solution has to be found beyond receptors, on the level of second messenger signal transduction and/or metabolic path(s).

Paškulin and coworkers showed the stimulating influence of ibogaine on rat brain energy metabolism, which results in the elevation of the enzymes of glycolysis and tricarboxylic acid (TCA) cycle and subsequent higher metabolic turnover[36]. On the other hand, experiment performed on yeast S. cerevisiae showed that induction of energy metabolism-related enzymes was not mediated through receptor bindings and it is not linked to cell differentiation or organization in tissue[35]. Yeast incubation with ibogaine was followed by a fall in ATP level and an increase in CO_2 production in a dose-dependent manner, suggesting that ATP consumption is induced by ibogaine[34, 35]. Beside the induction of energy metabolism-

related enzymes, SOD protein was also induced, suggesting that ibogaine has a role as pro-antioxidant in increased energy production and antioxidant cellular protection[34, 35].

Considering that ATP depletion affects the cellular mitochondrial metabolism, our unpublished data in erytrocytes as model system not dependant on mitochondrial energetics, showed dose-dependent increase of ATP in the blood plasma after 10 minutes of incubation. Doses applied in this experiment could influence membrane ATP pools, but not through changes in membrane fluidity[32]. Ibogaine as a lipophilic molecule freely enters the cell[34] and our unpublished results also demonstrated that ibogaine caused dose-dependent relaxation of both spontaneously and calcium-induced smooth muscle contractions of isolated rat uterus, followed by changes in antioxidative activity in the tissue[33]. The change is probably caused by transient dose-dependent fall in ATP level in the first hours of exposure to ibogaine[35], which entails immediate elevation of reactive oxygen species.

Conclusion

Despite complex pharmacological mode of action, it seems that the effects of ibogaine are mediated through its influence on energy metabolism and redox-active processes where ibogaine acts as pro-antioxidant. Further explanation of ibogaine pharmacology could elucidate its diverse effects, which would allow ibogaine to return to the list of medicaments for treatment of addiction.

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Molecular mechanisms of CNS response to inflamation – experimental and clinical studies results

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Neuroinflammation is a complex process with multiple mediators, signaling pathways, and feedback loops, which comprises the activation of glial cells, recruitment of peripheral immune cells, and the production of cytokines. Chronic overactivation of microglia leads to the consequent overproduction of reactive oxygen and nitrogen species which exert cytotoxic effects, promoting the pathogenic cascade and leading to serious changes and damage of neurons. An important role of oxidative and nitrosative stress in neuroinflammation and the severity of the clinical expression of the disease has been proved in our experimental (experimental autoimmune encephalomyelitis) and clinical studies (clinically isolated syndrome and multiple sclerosis patients). The intensity of oxidative and nitrosative stress parameters' changes significantly correlated with the clinical expression and radiological activity of the disease. The obtained results also point out that the modulation of NO synthesis can be of benefit in neuroinflammation, placing emphasis on methylated arginine derivatives as possible targets.

Introduction

Multiple sclerosis (MS) is an autoimmune chronic inflammatory disease of the central nervous system (CNS), afflicting mainly female young adults, accompanied with disability, resulting presumably from neuroinflammation. The pathological hallmarks of MS include blood-brain barrier (BBB) damage, T cell migration into CNS and their activation, demyelination, gliosis, and axonal/neuronal degeneration1. Although there are numerous hypotheses about the origin of CNS inflammatory responses, leading to neurodegeneration in patients with MS, the etiology of MS still remains poorly understood. The involvement of environmental factors and susceptible genes in disease pathogenesis has already been postulated, but the precise mechanisms of inflammatory cascade and CNS response interactions are still not clearly understood. Also, it is now well accepted that MS is not only an inflammatory disease[2] and that neuroinflammation and neurodegeneration are coupled processes, not only

in MS, but in many other pathologies affecting the CNS[3,4], there is still an open question whether inflammatory processes precede neurodegeneration or it is the opposite. Since grey matter lesions and brain atrophy have been found in these patients early, at the onset of the disease, the elucidation of the molecular mechanisms underlying neuroinflammation and neurodegeneration in MS is important to support new therapeutic strategies focused on neuroprotection in the early phase of the disease.

Neuroinflammation and neurodegeneration

Neuroinflammation is a complex process with multiple mediators, signaling pathways, and feedback loops. It comprises the activation of glial cells, recruitment of peripheral immune cells, and the production of cytokines, such as interferon-gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α)[5]. TNF-α mediates cytotoxic damage to glial cells and neurons, while IFN- γ induces cell surface molecules, important in immune and brain cells interactions[6]. Reactive glia shift toward proinflammatory phenotype, thus releasing cytokines, chemokines, and neurotoxic molecules[7], affecting neuronal injury and death through the production of neurotoxic factors like glutamate, S100B, TNF- α, IL-1β, prostaglandins, and reactive oxygen and nitrogen species.

As disease progresses, inflammatory secretions engage neighboring cells, including astrocytes and endothelial cells, resulting in amplification of inflammation, leading to neurodegeneration. Bender et al.[8] documented that the increased levels of TNF-α and IL-1β could alter the activity of neurons. Using primary rat and human neuronal cultures, Ye et al.[9] proved that these two proinflammatory cytokines induced cell death and apoptosis in vitro. Brain damage is accompanied by astrocytic activation that is characterized by the upregulation of glial fibrillary acidicprotein (GFAP) and proliferative and morphological alterations. Astrocytes are the most abundant glial cells in CNS with multiple roles in maintaining brain functions[10], regulating local CNS blood flow in response to changes in neuronal activity[11]. Beside, astrocytes produce a number of molecules, such as nitric oxide, prostaglandins, andarachidonic acid, that blood flow[12]. An altered interaction of astrocytes with the endothelial cells may also underlie the process of neuroinflammation in MS. When become activated, astrocytes produce a number of inflammatory mediators, such as chemokines, which attract inflammatory cells into the brain parenchyma[13].

Oxidative and nitrosative stress in neuroinflammation

Chronic overactivation of microglia and reactive oxygen species (ROS), produced by microglia or other cells in surrounding environment, have a damaging effects on neurons, but also modulate the activity of microglial cells. Beside, TNF- α can induce an additional release of ROS, by inducing NADPH oxidase activity[14]. In MS, CNS inflammatory process typically occurs as a condition that initiates (first clinical attack) and also exacerbates existing focal demyelinated lesions (relapses) created by activated microglia, as well as macrophages[15]. The consequent overproduction of reactive oxygen species and reactive nitrogen species (RNS) exert cytotoxic effects, promoting the pathogenic cascade and leading to serious changes in CNS[16].

In CNS, elevated levels of nitric oxide are associated with a number of inflammatory and neurodegenerative diseases, including multiple sclerosis[17]. Proinflammatory cytokines, chemokines and enzymes generated by a number of cells, including microglia, produce NO and reactive oxygen species that promote damage of oligodendrocytes and axons. At the same time with the cognition of NO critical role in the inflammatory process, a lot of studies have investigated its participation in the mechanisms of MS onset and development[18]. There is a number of mechanisms by which NO may contribute to nerve tissue damage under inflammation. Thus, NO causes vasodilation which generally occurs in conjunction with a disturbance of the permeability of the blood brain barrier, which promotes the passage of inflammatory cells[19] and mediators into the CNS parenchyma[20]. NO reactive species (nitrate, nitrite, peroxynitrite) promote stress disorders - myelin and nerve tissue destruction. It is supposed that NO provokes block in the action potential transmission, since NO donors occur as a reversible blocking agents in normal and demyelinating conditions, not only in the central, but also in the peripheral nerve system[21].

Several studies have documented the importance of NO in neuroinflammation and the pathogenesis of MS[22.23.24]. The best animal model to study the mechanisms of CNS changes in neuroinflammation, seen in MS patients, is autoimmune encephalomyelitis (EAE), and the majority of the current knowledge in this area has been obtained from these kinds of experiments. The results from our laboratory have proved the presence of oxidative and nitrosative stress in the pathogenesis of EAE, showing that inflammation, which is the base of the early EAE stage, might raise ROS and RNS levels (Fig 1.), leading to oxidative and nitrosative stress[25,26]. An important role of oxidative and nitrosative stress in the EAE pathogenesis and the severity of the clinical expression of the disease has been confirmed by the application of N-acetylcysteine (antioxidant agent) and aminoguanidine (nitric oxide synthase inhibitor), which exerted protective effects regarding clinical expression of EAE (Fig. 2)[27,28].

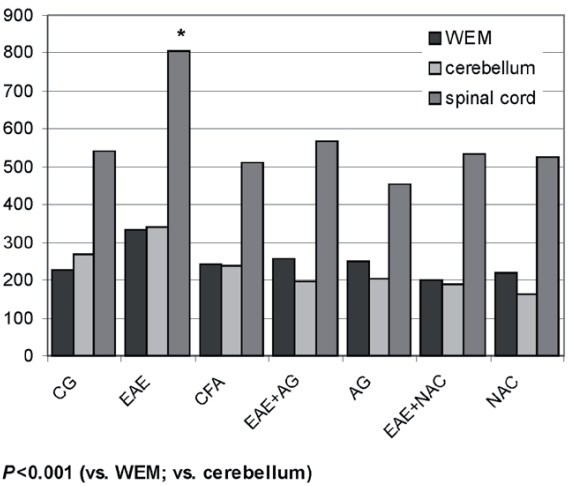

Figure 1. NO_2 and NO_3 conc. (nmol/mg prot.) In rat CNS different regions. (CFA) Complete Freund's adjuvant; (AG) aminoguanidine; (NAC) N-acetyl-L-cysteine; (CG) control group; (EAE) rats with expe-rimental autoimmune encephalomyelitis; (CFA) CFA treated rats; (EAE+AG) EAE rats treated with AG; (AG) rats treated with AG;(EAE+NAC) EAE rats treated with NAC; (NAC) rats treated with NAC. weakness;

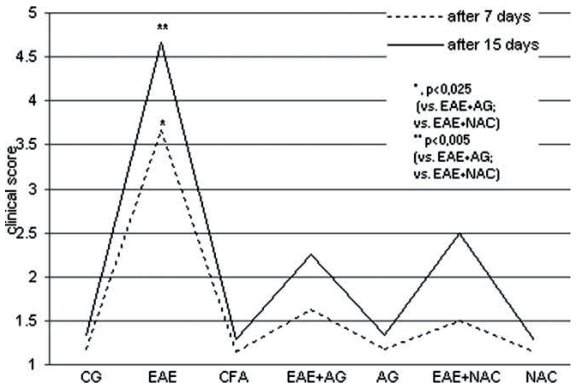

Figure 2. The clinical score of neurological disorder (1–6) of rats after 7 and 15 days. CG - control group; EAE - rats with experimental autoimmune encephalomyelitis; CFA – rats treated with complete Freund's adjuvant; EAE + AG – EAE rats treated with aminoguanidine; AG – rats treated with aminoguanidine; EAE + NAC – EAE rats treated with N-acetyl-L-cysteine; NAC – rats treated with N-acetyl-L-cysteine. 1 – healthy rats; 2 – loss of tail tone; 3 – hindlimb 4 – hindlimb paralysis; 5 – hindlimb paralysis plus forelimb 6 – moribund or dead.

In the context of EAE clinical expression, NO• produced by iNOS activity plays a dual role. On one side, it is thought that it mediates protection, because EAE symptoms are exacerbated in iNOS-/- mice29. On the other hand, the data from our experiment on EAE iNOS-/- mice30 are consistent with the hypothesis that NO• has cytotoxic effects that include oligodendroglia disruption and an impairment of the ability of myelin supporting cells to maintain and produce myelin during an early EAE phase, since EAE clinical expression in wild type mice was more severe than in iNOS-/- mice.

Oxidative stress has been revealed as the main contributor in the pathophysiology of neuroinflammation. In our clinical studies, we have investigated oxidative and nitrosative stress markers in plasma and cerebrospinal fluid (CSF) of patients with different clinical phenotypes of neuroinflammation, defined as clinically isolated syndrome (CIS), and relapsing remitting multiples sclerosis (RRMS)[31]. We have analyzed oxidative stress intensity during these neuroinflammatory acute attacks in regard to the clinical and radiological features of CNS inflammation. The obtained results showed an increase in plasma and CSF malondialdehyde levels in both CIS and RRMS patients compared to control values, which significantly correlated to the severity of their clinical presentations. Also, in both groups of studied patients, the positive correlations were observed in MDA levels in plasma and CSF and their radiological findings.

The reactions, initiated by the reactive species, include peroxidation of lipids, nitrosylation of thiol groups, nitration of tyrosine, and the oxidation and deamination of nucleic acids. The results of all our clinical studies in MS patients revealed increased oxidative and nitrosative stress parameters in plasma[31,32] , which were effectively corrected by 18 and 30 month interferon-β 1b (IFN- γ1b) treatment[32]. Prooxidative processes, as the results of immune cells activity, cause oxidative modification of many biological macromolecules (proteins, lipids, and nucleic acids), which are responsible for pathological and clinical features of a variety of neurodegenerative diseases, such as MS, which was also documented

in our clinical investigations[32,33]. Therefore, the control of ROS/RNS and their cytotoxic bioproducts in affected neuronal cells, through scavenging enzymes or thiol repair enzymes, play the main role in nerve tissue repair, protection and survival[34]. The decreased plasma and erythrocyte antioxidant capacity in MS patients in our studies[35,36] is in accordance with this hypothesis of Witherick et al[34]. The measured activities of catalase and total superoxide dismutase in plasma of CIS and RRMS patients were significantly higher compared to the control values, while an increased catalase activity and the decrease in superoxide dismutase activity, regarding the values obtained in control group were found in CSF. The positive correlations, regarding clinical score, were obtained for all tested biomarkers[31].

Taking in account the cognition of NO critical role in inflammatory process, as well as the presence of nitrosative stress in MS patients documented in our clinical studies[32], we came to an idea to investigate the parameters of L-arginine metabolism in these patients. Nitric oxide is synthesized from L-arginine in the reaction catalyzed by the nitric oxide synthases (NOS) family: neuronal (nNOS, NOS1), endothelial (eNOS, NOS3) and inducible (iNOS, NOS2). The metabolism of L-arginine is determined by the expression of the arginine metabolizing enzymes iNOS and two arginase isoforms, arginase I and II. In all our study patients, arginase activity showed the decreased values compared to controls[32,37]. The possible explanation of the obtained data could be found in the current knowledge about the arginine degrading pathways, which suggests iNOS induction by T helper I cytokines (IL-1, TNF- α and INF-γ), more intensive in acute attacks of CNS inflammation, while arginase is induced by T helper II cytokines and other immune regulators, such as IL-4, 10, and 13, TGF-β and prostaglandin E2[38]. These findings explain the absence of arginase-mediated downregulation of NO formation via above-described mechanisms and are in accordance with the increased NO production.

Furthermore, through polyamine synthesis and the limitation of L-arginine availability in the extracellular environment, arginases can modulate macrophage and T-lymphocyte activation so far regulating the intensity of inflammation[38]. The results of Ahn et al.[39,40] suggest that the increased level of arginase I in EAE and spinal cord injury is associated with an increase in macrophages and reactive astrocytes, possibly contributing to the modulation of inflammation during the course of disease. Although arginase seems to be important to the control of effector immune cells, there are also reports suggesting that iNOS, but not arginase activity induction can limit the autoreactive T cells functions, reducing autoimmune diseases severity[41]. There are also opinions that iNOS and arginase may be co-induced in immunostimulated macrophages42.

The results from our studies suggest that arginase is an important contributing enzyme in neuroinflammation, taking in account the increase of arginase activity after 18 and 30 months of IFN- γ 1b treatment which was in correlation with the patients' EDDS score[32], although, probably, its effects are only partially mediated through the NO pathway,. The lower arginase activity in our study patients with higher EDSS and negative correlation between enzyme activity and severity of clinical disease expression, points out the importance of immune

intensity regulation via arginase-mediated mechanisms. It has also to be mentioned that arginase activity was significantly higher in RRMS compared to CIS patients, which is in accordance with literature data that in the later phase of inflammatory process, which is characteristic for RRMS patients, iNOS activity is decreased, whereas arginase is highly induced[43]. The correlations between enzyme activity and Gd enhancement brain lesion volume are in accordance with this. The obtained results suggest that the modulation of NOS and arginase activity can be of benefit in the treatment of neuroinflammation and that NOx concentration and arginase activity in CSF and plasma might serve as markers of clinical and radiological activity of neuroinflammation.

The possibility of NO synthesis modulation in neuroinflammation directed our attention to methylated arginines. Methylated arginine derivatives, symmetric dimethylarginine (SDMA) and asymmetric dimethylarginines (ADMA) are produced by the methylation of protein arginine residues in the reaction catalyzed by arginine methyltransferases (PRMT). During proteolysis, methylated arginines are released and do not remake again in translation process. ADMA is the major endogenous NOS inhibitor and, also a competitive inhibitor of the cellular L-arginine uptake[44]. Plasma concentration of ADMA in healthy people is lower than 1 μmol/L. In our study, MS patients' plasma ADMA and SDMA levels are meaningly low, even during the remission period[32]. ADMA and SDMA levels decreases may be the consequences of arginine methyltransferases covalent modification induced by an intensive nitrosative and oxidative stress[25,27].The found changes are in correlation with the increased levels of NO derivatives and, at the same time, offer a possible explanation for the increased NO production. These findings are also in strong relation with patients' clinical states. In addition, INFβ1b therapy significantly increased ADMA plasma levels and decreased nitrate and nitrite levels, which was in correlation with the patients' EDSS scores[32].

The other methylated arginine derivative, SDMA, is a stereoisomer of ADMA, produced in equivalent quantities. Although it does not inhibit NOS, SDMA may compete with L-arginine for cellular uptake, thus limiting this substrate availability for NOS and promoting amelioration of neuronal tissue damage and disease clinical signs[45]. Also, in neuroinflammation, due to produced proinflammatory cytokines, such as TNF-α, ADMA is accumulated in nerve cells, which is a possible explanation for the positive effect of TNF-α in the acute phase of the immune mediated diseases[44,46]. Based on the presented data, ADMA and SDMA appear to be the potent modulators of NO synthesis and the inflammatory response in nerve tissue damage during acute phase of MS .

Conclusion

The existing protocols for MS treatment, modified regarding to disease species, show respectable results, besides a wide variety of therapy responses. But, the recent studies have shown therapy response heterogeneity, not only between different MS subtypes, but also between patients with the same subtype of the disease.

The results from our laboratory have proved an important role of oxidative and nitrosative stress in the pathogenesis of EAE (neuroinflammation), as well as CIS and MS. The correlation between the results from clinical studies and experimental ones provide an evidence of the important role of redox homeostasis in neuroinflammation and help in the identification of new promising targets to control neuroinflammation through redox control in these patients.

In conclusion, the presented data suggest that the determination of NO-related metabolites in plasma may be valuable in the clinical monitoring of MS patients, also providing information about the choice of therapy intervention, focused on concrete pathogenetic factors in each individual patient. Also, these findings might be useful in providing the earliest antioxidative treatment in neuroinflammation aimed to preserve total and CNS antioxidative capacity parallel with delaying irreversible, later neurological disabilities. Also. the arginine methyl derivates, as NOS inhibitors, certainly deserve the attention as potential biomarkers and the focus of future investigations.

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MIF – a novel modulator of insulin activity

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Insulin is a hormone that primarily regulates glucose homeostasis, but also exerts several other functions such as increase of DNA replication and protein synthesis via control of amino acid uptake and modification of the activity of numerous enzymes. Insulin undergoes a complex process of post-translational modifications that finally results in formation of bioactive insulin. Although the mechanism of insulin production is almost entirely discovered, we have identified macrophage migration inhibitory factor (MIF) as previously unknown modulator of insulin biosynthesis. It is firmly established that MIF deficiency has a negative impact on insulin activity. However, the quantity of insulin production and the rate of its secretion are not altered in the absence of MIF. Yet, we have found that MIF binds insulin. Since it has been proved that MIF's enzymatic function is indispensable for insulin activity and that the presence of MIF favors stable insulin hexamer formation, we propose that MIF might act as adaptor protein that enables aggregation of insulin monomers. This newly discovered feature of MIF could be potentially important for commercial production of insulin, for increasing its stability and/or bioavailability.

Introduction

Insulin is a peptide hormone that regulates glucose metabolism. The first experiments that led to insulin discovery was performed by Paul Langerhans in 1869 who noticed small clumps of cells within the pancreatic tissue and named them pancreatic islets or islets of Langerhans. The relation between the pancreas and glucose metabolism was first noticed by Oscar Minkowski and Joseph von Mering, but the actual discovery of insulin as an active entity in maintaining glucose homeostasis was made by Frederick Banting and John James Rickard Macleod for which they were awarded the Nobel Prize in Physiology or Medicine in 1923[1].

The production and regulation of insulin secretion is a very complex process as it involves actions of many enzymes, receptors, ionic pumps and so on. Although this process has been precisely described both for the sake of the basic science and also for commercial insulin production, we propose that macrophage migration inhibitory factor (MIF) may be a new piece of insulin biosynthesis puzzle.

Insulin production

Insulin is produced in beta cells of pancreatic islets. It is first synthesized as an inactive protein called preproinsulin that contains signal peptide necessary for its entrance into the endoplasmic reticulum (ER) for post-translational processing. Within ER, the signal sequence is proteolytically removed to form proinsulin that further undergoes formation of three vital disulfide bonds and acquires a proper conformation. From ER, proinsulin is transported into the lumen of Golgi apparatus where further post-translational modifications occur. Proinsulin consists of three domains: an amino-terminal B chain, a carboxy-terminal A chain and a connecting peptide in the middle known as the C peptide. Since active insulin consists of A and B chains only, proinsulin is being cleaved within Golgi by the action of cellular endopeptidases known as prohormone convertases (PC1 and PC2), as well as the exoprotease carboxypeptidase E. Mature insulin is stored in the secretory granules in the inactive form of hexamers (six molecules of insulin linked with Zn^{2+})[2].

Insulin secretion

Upon various stimuli, primarily elevated glucose concentration in the blood, beta cells secrete insulin by exocytosis. The process of insulin secretion is tightly regulated. For glucose sensing, beta cells use glucose receptors GLUT2 that enable transport of glucose from the blood into the cell. Glucose is phosphorylated by the rate-limiting enzyme glucokinase that enables further degradation of glucose by glycolysis and Krebs cycle when multiple, high-energy ATP molecules are created, leading to a rise in the intracellular ATP: ADP ratio. These events lead to the closure of the ATP-sensitive SUR1/Kir6.2 potassium channel resulting in an increase in K^+ and positively charged cytoplasm. As a result, depolarisation of the cell surface membrane occurs and voltage-gated Ca^{2+} channels open to allow Ca^{2+} facilitated diffusion into the cells. An increased intracellular Ca^{2+} concentration causes the activation of phospholipase C, which cleaves the membrane phospholipid phosphatidyl inositol 4,5-bisphosphate into inositol 1,4,5-trisphosphate and diacylglycerol (IP3). After binding of IP3 to receptor proteins in the plasma membrane of the ER, Ca^{2+} leak into the cytoplasm from the ER and further raise the intracellular concentration of Ca^{2+}. The increase in intracellular Ca^{2+} concentrations triggers fusion of the insulin-storing granules with the plasma membrane and expulsion of insulin hexamers. Dissociation of the hexamers to monomers and their transport to the liver and other tissues then occurs via the blood stream. Because the monomer is exquisitely susceptible to fibrillation[3], its zinc-mediated assembly within beta cells may represent a defense against toxic misfolding in the secretory granule[4]. Insulin action occurs after the binding to the insulin receptors what triggers both Ras→MAP kinases and PI-3K→Akt signaling cascades[5] and finally transcription of genes whose products activate glucose transport into the cell.

Insulin granules content

In addition to insulin, beta cell secretory granules contain at least 50 polypeptides[6], some of which have biological functions like islet amyloid polypeptide and chromogranin A. The granules also store a number of low-molecular weight compounds like ATP, GABA, serotonin and glutamate and high concentrations of Zn^{2+} and Ca^{2+} ions[7]. Interestingly, Weaber et al., in 1997[8] found that the granules contain macrophage migration inhibitory factor (MIF), homotrimer that might play an important role in insulin biosynthesis (Figure 1).

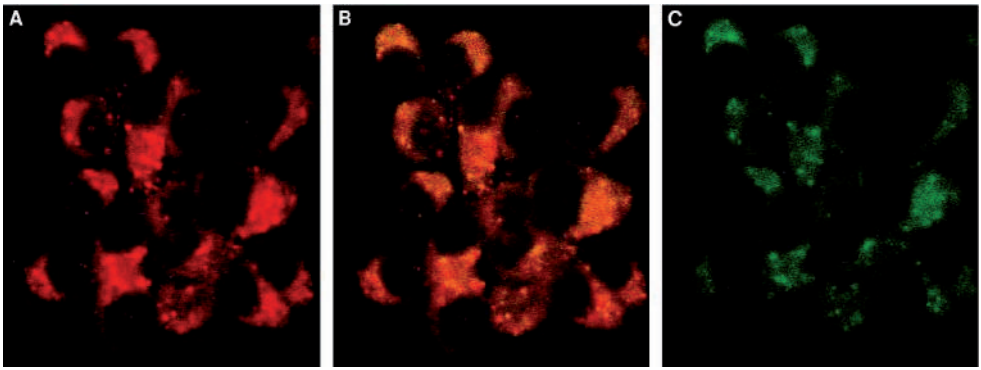

Figure 1. Beta cells stained with anti-insulin antibody (red), anti-MIF antibody (green) and their co-localization (orange)[8].

MIF – a pleiotropic molecule

MIF possesses a number of functions such as modulation of the immune response when it acts as a pro-inflammatory cytokine, then it may act as an enzyme[9], endocrine molecule[10] and a chaperon-like protein[11]. MIF is expressed in a variety of cell types including epithelial, endothelial and immune cells[12]. In addition, MIF is highly expressed in several tissues of the endocrine system including hypothalamus, pituitary, adrenal glands and islets of pancreas[13]. Unlike other cytokines, MIF is constitutively expressed and stored in intracellular pools and does not require de novo protein synthesis before secretion[14].

MIF and diabetes

MIF has been implicated in pathogenesis of many inflammatory and/or autoimmune diseases including type 1 diabetes (T1D) and type 2 diabetes (T2D)[15,16]. Increased MIF serum level is associated with development of T1D, obesity and insulin resistance[17-19]. In animal models of diabetes, MIF inhibition or deletion prevents spontaneous or chemically-induced autoimmune diabetes[20,21]. Furthermore, the absence of MIF protects pancreatic islets from

detrimental influence of cytokines and high level of nutrients[22,23]. Although MIF's role in diabetes pathogenesis is usually examined from the immunological point of view, current knowledge indicates that MIF directly modulates insulin secretion and glucose metabolism and that treatment of pancreatic islets with anti-MIF antibody reduces glucose-stimulated insulin release[8]. Moreover, MIF is secreted together with insulin and it acts as an autocrine factor to stimulate insulin release[8]. The absence of MIF in MIF-knockout mice (MIF-KO) results in the development of obesity, glucose intolerance at 6 months of age and consequent hyperglycemia[24]. On the other hand, these mice are sensitive to administration of exogenous insulin indicating that receptor for insulin is fully functional as well as insulin-triggered signaling pathway in target cells[24]. Having this in mind, it could be speculated that the explanation for the observed condition in MIF-KO mice may reside either in the improper function of beta cells or reduced insulin activity.

MIF and beta cell function

As MIF-KO mice exhibit impaired glucose tolerance i.e. inability to lower blood glucose after exogenous glucose administration, it can be hypothesized that MIF-deficient beta cells do not function properly. Seemingly this is not the case since MIF-deficient beta cells express equal amounts of insulin mRNA as WT (C57BL/6) beta cells do. Moreover, MIF-KO beta cells produce and secrete comparable levels of insulin to WT islets thus proving that MIF absence does not influence the function of beta cells[25].

MIF and insulin activity

The activity of insulin can be estimated by its ability to drive glucose translocation into the cell upon binding to insulin receptor. Very interesting finding is that insulin derived from MIF-KO pancreatic islets almost completely fails to trigger glucose uptake into the hepatocytes. The reduced MIF-KO insulin activity is also confirmed by lower Akt phosphorylation in hepatocytes, a key downstream molecule in insulin receptor signaling pathway[25].

How does MIF contribute to insulin bioactivity? It is completely certain that MIF's tautomerase activity is not crucial for insulin function since both ISO-1 and vitamin E (inhibitors of MIF tautomerase function) are unable to alter insulin function. Further, although the molecular weight of WT (isolated from islets of C57BL/6 mice) and MIF-KO-derived insulin is the same, MIF-KO-derived insulin showed higher susceptibility to trypsin digestion than WT insulin indicating that MIF-KO insulin has a different protein conformation compared to WT insulin. And finally, the most important finding is that MIF favors insulin hexamer formation in cell-free circumstances thus far suggesting that it can act as an adaptor protein for insulin oligomerization[25]. The crucial event in insulin biosynthesis is formation of hexamers within the granules since the presence of monomers triggers their association into completely inactive fibrils[26]. So far, the sole responsible factor for hexamer formation has been Zn^{2+}. However, when these results are taken into account, it could be

hypothesized that MIF might have the same role as Zn^{2+}. To prove this, both recombinant proteins were dissolved in phosphate buffer saline (a solution that does not contain Zn^{2+}) and formation of insulin hexamers occurred just the same.

Does MIF bind insulin?

As it is previously mentioned MIF co-localizes with insulin in the secretory granules of beta cells. Consequently, one question arises: does MIF physically interact with insulin? Using a software modeling, we have found that there is at least one probable binding site with very low binding energy as shown in Figure 2[25]. These empirical data are confirmed in cell-free conditions where we have found by immunorecognition that insulin covers immunodominant MIF epitope. What is more, immunoprecipitation studies revealed that MIF binds to insulin within beta cells suggesting that their interaction is a way how MIF modifies insulin and helps acquiring its full activity[25].

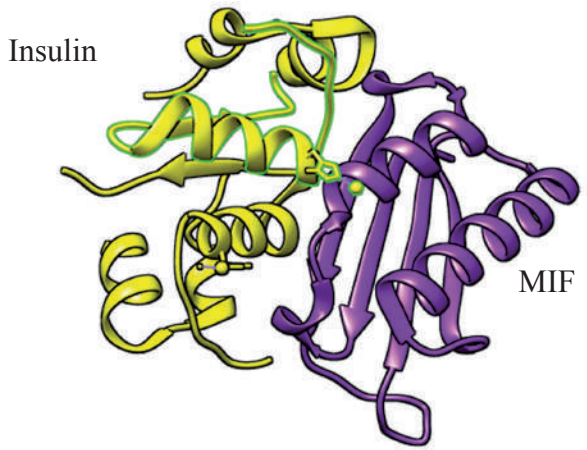

Figure 2. Interaction between MIF (purple) and insulin (yellow) obtained by using FireDock software[25].

Conclusion

Our study shows that MIF, a molecule with primarily immunological features, physically interacts with insulin thereby changing its conformation and promoting formation of stable hexamers. Since hexamers are inactive form of insulin it still remains unclear how MIF enables insulin's full bioactivity. One possible explanation might be that the role of MIF is to prevent insulin monomers aggregation into entirely inactive fibrils. Therefore, the discovery of MIF as an important contributor in insulin biosynthesis might have serious implications for commercial insulin production.

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Molecular analysis of thyroid gland tumors

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For many years fine-needle aspiration (FNA) has been used for evaluation of thyroid nodules, and the FNA cytology result is the primary determinant whether thyroidectomy is indicated or not. When cytology is indeterminate, additional molecular analysis is required. The main goal of molecular diagnostics of thyroid nodules is to prevent the unnecessary surgery in patients with benign nodules. This article focuses on the molecular markers suitable for differentiating benign from malignant thyroid nodules, including their advantages and limitations.

Performing molecular analyses such as: somatic mutation testing, gene and miRNA expression, and immunocytochemistry enables patients to be separated into low-risk and high- risk categories. Somatic mutation tests have a high specificity, i.e. when mutation is detected, the risk of malignancy is high, but when the test is mutation negative, overall malignancy cannot be excluded. Gene expression platforms have proven successful in detecting benign nodules, but are suboptimal for detecting a malignancy. MiRNA expression via RT-PCR is an achievable and promising method to detect indeterminate thyroid lesions, has several advantages over gene expression, but the consensus about the the panel of miRNAs to be tested, is still to be established. Immunocytochemistry of Gal-3, HBME-1, CK-19 and other markers have been evaluated in indeterminate thyroid nodules, but the range of reported accuracy is wide. Thus all considered the selection of the best molecular markers and the most favorable combination of markers is still a matter of debate.

Introduction

Incidence rates of thyroid cancer are still increasing among both men and women[1]. The risk factors that contribute to its development are iodine deficiency, female sex, increasing age, smoking, exposure to radiation and a thyroid cancer family history[2-4].This cancer is typically manifested as a thyroid nodule. Palpable nodules are found in about 4 to 7% of the population, while nodules that can be detected by ultrasound (sonography) are present in 19 to 35% of the population, and nodules are discovored at autopsy in 8 to 65% of people[5]. The incidence of malignancy is up to 13% for solitary nodules, and up to 10% for multinodular goiter[6-8].

The first step in evaluation of solitary thyroid nodules is ultrasonography (US), followed by fine-needle aspiration (FNA). Several recently published studies indicate that the use

of conventional ultrasonography does not allow accurate prediction of the histology of solitary thyroid nodules[9-11]. The main indications of US evaluation are measurement of nodule size, assessment for possible lymphadenopathy, and use as a guide for FNA.FNA has been employed for many years for examining of thyroid nodules, and cytology of the aspirates is the primary determinant for whether thyroidectomy is indicated. According to the Bethesda classification, cytological diagnosis of thyroid nodules can be categorized as: (I) non-diagnostic or unsatisfactory, (II) benign, (III) atypia of undetermined significance or follicular lesion of undetermined significance (AUS/FLUS), (IV) follicular neoplasm or suspicious for follicular neoplasm (FN/HCN), (V) suspicious for malignancy, (VI) malignant[12].

The most problematic of the above mentioned categories, in terms of decision making, are the indeterminate categories (Bethesda III, IV, and V). Roughly, between 15 and 30% of thyroid nodules fall into an indeterminate category, not being not clearly benign or malignant. The risk of malignancy for these categories falls between 5 and 75%[12]. When the cytology is indeterminate, patients are subjected to a partial gland removal. For more than half of these patients, the pathology results are benign, so, these patients had unnecessary surgery. On the other hand, if the results are malignant, these patients have to be operated on once more, in order to remove the rest of the thyroid gland. However, in addition to cytology, molecular testing can be useful in assisting to estimate the cancer risk.

The main goal of molecular diagnostics of thyroid nodules is to prevent the unnecessary surgery in patients with benign nodules, and also to prevent patients with malignant nodules from being subjected to a repeat operation. Moreover, in cases of indeterminate cytology, it has been decided that the risk of watchful waiting might be too high for most patients[13].

During the last 8 to 10 years, different approaches for preoperative molecular testing have arisen. In terms of benefits and costs, the molecular testing approach has been shown both to save money, and to prevent exposure of patients to the risk of an operation[14].

The focus of this article is directed towards molecular markers suitable for differentiating benign from malignant thyroid nodules, together with their advantages and limitations.

Molecular markers used for evaluation of thyroid nodules

1. Somatic mutations

Two signaling pathways are considered to be the most important for the development of thyroid cancer, the mitogen-activated protein kinase (MAPK) pathway and the phosphatidylinositol 3'-kinase (PI3K)-Akt pathway. MAPK activation is crucial for tumor development. If one of the signal transducers in this pathway, such as BRAF or Ras, carries a mutation, the MAPK is constitutively activated, resulting in changes in the expression of genes that regulate important cellular processes, such as cell growth, proliferation and survival[15].

BRAF mutation is one of the most prevalent somatic genetic events in human cancer and has the hallmark of a conventional oncogene[16]. The most common BRAF mutation found in

thyroid cancer is V600E (>95%)[17]. This mutation leads to a constitutive activation of a BRAF kinase and is the most common genetic change in papillary thyroid carcinoma (PTC). The V600E mutation is also found in poorly differentiated and anaplastic carcinomas, suggesting that it occurs early in mutagenesis, and that these cancers are PTC derived[18]. Other rare BRAF mutations have been described, such as K601E, small in-frame insertions or deletions surrounding codon 600[19], but they are present in a minority of papillary carcinomas 1-2%[20]. Besides point mutations, the BRAF gene can also display copy number variation due to amplification of the gene, or gain of one or more copies of chromosome 7[21]. Another BRAF gene alteration is AKAP9/BRAF rearrangement. The AKAP9/BRAF fusion gene lacks the regulatory domain of BRAF. This results in a constitutively activated kinase, similar to that of the V600E variant[22]. AKAP9 provides an active promoter that drives the expression of truncated BRAF. It is still unclear whether AKAP9 itself contributes independently to the transforming potential. AKAP9/BRAF rearrangement is common in carcinomas with radiation exposure.

Another important group of genes whose mutations are responsible for the thyroid cancerogenesis is the Ras gene family. Ras oncogenes are G-proteins, residing on the inner side of membranes, and they transmit signals from a variety of growth factor receptors along the MAPK and PI3K pathway[23]. Three genes belonging to the Ras family have a somatic mutation linked to a cancer diagnosis: NRAS, HRAS and KRAS, and the mutation hotspots are localized in the GTP binding domain (codon 12/13) or the GTPase domain (codon 61) in all three genes[24]. Ras mutation occurs most frequently in follicular adenoma, the follicular variant of PTC, follicular carcinoma, and is highly prevalent in poorly differentiated and anaplastic carcinomas[15]. Ras mutations are the second most common mutation detected in FNA samples from thyroid nodules, and have 89-97% positive prediction value for malignancy[25,26].

RET/PTC is a genetic rearrangement formed when a 3' portion of the RET gene tyrosine kinase domain is fused to the 5' terminal sequence of another genes, leading to constitutive activation of the RET gene[27]. Several forms of resulting chimeric oncoproteins have been discovered, but the two most common rearrangements are: an H4 - RET translocation resulting in a RET/PTC1 and ELE1 - RET resulting in a RET/PTC3 fusion gene[28]. This form of genetic rearrangement is found in approximately 19% of papillary carcinomas and is present both in naturally occurring and in radiation-induced tumors[27]. The prevalence of the RET/PTC mutation varies greatly, and this can be attributed to sensitivity of different methods used to detect it and to the heterogeneous distribution of this rearrangement within tumors[29]. Therefore, it is safe to say that the prevalence of this mutation in papillary carcinoma is between 10 and 20%[15].

The PAX8/PPARγ translocation fuses the thyroid specific transcription factor PAX8 gene with the PPARγ gene, which is a transcription factor that regulates genes involved in adipocyte differentiation and lipid metabolism[30]. This mutation is most prevalent in follicular thyroid carcinoma, where it occurs with a frequency of 30-35%[31]. Its presence in FNA samples is not diagnostic for malignancy, but it indicates the necessity of a search for vascular or capsular

invasion on histological specimens[32].The text below provides a brief overview of studies where mutation testing of FNA samples was used for cancer diagnostics.

A pioneer study in this area, conducted by Prof. Nikiforov, was based on the finding that certain mutations are strongly correlated with the presence or absence of thyroid cancer[26,33]. A panel of mutations was selected, comprising four point mutations: BRAF, NRAS, KRAS and HRAS, and three genetic rearrangements: RET/PTC1, RET/PTC3 and PAX8/PPARγ. The use of molecular testing of FNA enabled significant cost reduction, compared to lobectomy or total thyroidectomy[14]. In a prospective study of 1056 FNAB samples with indeterminate cytology[33], analysis of the mutation panel gave a positive predictive value ranging from 87-95% for thyroid cancer. In a group of mutation negative cancers, all with indeterminate cytology, the cancer risk was only 11%. BRAF V600E, PET/PTC, PAX8/PPARγ mutations were associated with malignancy in nearly all nodules. The Ras mutation indicated a lower risk of thyroid malignancy, 85%, because it occurs in follicular adenomas as well as in carcinomas.

On the other hand, Kleinman et al.[34] claim that the molecular analysis does not significantly alter the initial operation management for the patients with indeterminate cytology. The sensitivity of cytology alone was 73%, and for cytology combined with the V600E mutation it was 76%. This study showes that single mutation screening is not sufficient to improve preoperative risk stratification.

Filicori and associates analyzed the indeterminate lesions found in different studies that had been tested for BRAF, RET, RAS and PAX8/PPARγ mutations[35]. In cases where FNAs were mutation negative, the risk of malignancy was still significant, i.e. 22%. This is similar to the malignancy rate of 20-30% in indeterminate lesions. When nine studies were taken together, 487 samples of FNA and the combined mutation panel resulted in 40% sensitivity and 98% specificity. When RAS was excluded, the specificity of the mutation testing was 100%.

Moses et al.[36] reported that the negative predictive value of molecular testing is still too low (65%) to eliminate diagnostic thyroidectomies. A mutation panel consisting of BRAF, KRAS, NRAS, RET/PTC1, RET/PTC3, and NTRK1, gave 38% sensitivity and 65% specificity.

Ohori et al, observed that in all patients with positive molecular markers carcinoma was diagnosed in the final histopathological diagnosis[37]. Indeterminate cytology lesions that were negative for mutations had a cancer outcome in 7.3% of patients. The authors suggested a combination of molecular analysis and repeated FNA sampling, which could increase the sensitivity of papillary thyroid carcinoma detection.

The presence of BRAF, RAS, RET/PTC, TRK and PAX8/PPARγ mutations was associated with cancer in 91% of cases and with adenomas in 8.9% of cases. Molecular analysis combined with cytology resulted in total accuracy of 93.2% compared to 83% for cytology alone. Cantara et al.[25] recommend that the presence of RAS mutation in follicular adenoma should be considered as a false positive result.

Eszlinger et al. established that the BRAF mutation and RET/PTC rearrangement coincide with cancer in all cases, while the presence of the RAS and PAX8/PPARγ mutations are associated with cancer in only 50% of cases[38]. The authors stress the need to include

additional somatic mutations or miRNA as markers in order to further improve the FNA diagnostics further.
Taken together, mutation studies increase diagnostic accuracy, due to their high specificity. Implementation of mutation analysis in addition to cytology evidently improves the management of thyroid nodules. The downside of this approach is that approximately 30% of thyroid cancers harbor none of the mutations tested in the above mentioned panels. Therefore, when the test is mutation negative, an overall malignancy risk is still present.

2. Gene expression analysis

This approach is based on the gene expression classifier, with the aim of proving that the nodule is benign, rather than demonstrating malignancy. The first study was published by Chudova et al.[39], where 247186 transcripts were analyzed in 315 thyroid nodules, and 48 FNAB samples, half of which had an indeterminate cytology. The mRNA expression of genes differently expressed in malignant and benign thyroid nodules was assessed. Sensitivity of 80 to 100% and specificity of 40 to 73% were obtained, for thyroid tissue and FNA samples, respectively.
Alexander et al.[40] used the same gene classifier as described above, with the interpretation of a novel diagnostic test that measured the expression of 167 genes, pre-selected based on their ability to distinguish benign from malignant changes. Out of these 167 genes, 142 were classifiers (benign or suspicious for malignancy diagnosis) and 25 had the role of excluding rare neoplasms. This test had a negative predictive value (NPV) higher than 95%, and thus enabled physicians to rule out malignancy and to prevent unnecessary surgery. The gene expression classifier is able to select a population of patients with a low cancer risk.
Prasad et al. tested the expression of 75 genes previously shown to be promising candidates for identifying cancer cells[41]. The combination of three genes: MRC2, HMGA2 and SFN was shown to be helpful in diagnosing thyroid cancer with 96% test specificity and 91% negative predictive value.
Another gene expression analysis study, using a real time quantitative RT-PCR multigene assay was designed by Kebebew and associates[42]. As markers, the expression of ECM1, TMPRSS4, ANGPT2 and TIMP1 was investigated, and correlated with tumor aggressiveness according to the TNM staging system and AMES risk classification system. This assay correctly classified 93% cancers into low and high-risk groups with a sensitivity of 79% and a specificity of 92%.
The foregoing studies suggest that gene expression analysis offers a wide pallet of genes that could be tested for a malignancy diagnosis. The platforms based on microarray analysis are successful in detecting benign nodules, nonetheless they still remain suboptimal for detecting malignancy.

3. miRNA analysis

MicroRNAs (miRNAs) are endogenous noncoding RNAs, formed as short hairpin oligonucleotides. They negatively regulate the expression by binding to the 3' non-coding region of a target mRNA, resulting in its degradation or the inhibition of translation[43]. One miRNA can have many different targets, and is often involved in feedback loops along with other regulatory factors[44]. miRNAs are suitable biomarkers because their expression is often dysregulated in cancer, and they are more stable than other RNAs, making isolation and quantification by qPCR more feasible[45].

Expression of miRNAs is altered in thyroid cancer, that is, normal thyrocytes have a unique miRNA expression profile and many miRNA are dysregulated in thyroid cancer. In further text, we will focus on those studies where miRNAs were used to differentiate benign from malignant nodules only in FNA samples with indeterminate cytology.

Thus, Nikforova et al.[46,47] examined only 8 out of 62 FNA samples that had indeterminate cytology. A set of seven miRNAs (miR-187, miR-221, miR-222, miR-146b, miR-155, miR-224, and miR-197) was pre-selected as the most differentially expressed between benign and malignant tissue. When the expression of one miRNA is at least doubly upregulated, the sensitivity of the test reached 100% and the specificity was 94%. When three or more miRNAs were upregulated, the sensitivity was 88% and the specificity was 98%.

One hundred and eighty one patients with indeterminate FNA cytology were included in the study of Keutgen et al.[48]. Evaluating a set of six different miRNAs as diagnostic tools for indeterminate thyroid nodules, the authors showed that when four out of the six miRNAs were combined, they accurately classified 90% of the patients with 100% test sensitivity and 86% specificity.

The prospective study of Agretti[49] on 141 FNA samples obtained from 138 patients, found statistically significant increase for six out of seven miRNAs in malignant FNA samples. Measuring the expression of only three miRNAs: mir-146b, mir-155 and mir-221, was sufficient to differentiate malignant from, benign nodules. Malignant vs. benign prediction was 60% valid for nodules with indeterminate cytology. Mir-146b was the most upregulated miRNA. In this panel, an accuracy of 59% with 11% of false negatives results was achieved.

Among a total 128 FNA specimens, Shen et al.[50] noted 68 with indeterminate cytology. A combination of four miRNAs (miR-146b, miR-221, miR-187 and miR-30d) was found to have 94% sensitivity and 95% specificity. Sensitivity of 63.6% and specificity of 78.9% were obtained for thirty cases with atypical cytology. These data imply that further refinement of the miRNA panel used for diagnosis of atypical nodules is needed. Moreover, this panel was inaccurate for differentiating follicular neoplasias from fvPTC.

The study of Kitano and associates[51] included only 21 indeterminate FNA samples and favored mir-7 as the best predictor of malignancy. When mir-7 expression was measured in the indeterminate FNAs, the accuracy dropped to 37%, sensitivity was 100% and specificity was 20%.

Keutgen et al. characterized 29 indeterminate FNAs[48]. A predictive model was established using four miRNAs: miR-222, miR-328, miR-197, and miR-21. When Hurtle cell neoplasms

were excluded, the panel of these four miRNAs reached 100% sensitivity and a specificity of 95% for differentiating malignant from benign indeterminate lesions.
Dettmer and associates[52] focused on conventional and classical follicular thyroid carcinomas, and they demonstrated how the expression of three miRNAs allowed distinction of malignant from benign forms of follicular thyroid carcinoma (FTC). An array of 381 human miRNAs was used for the analysis. Classification and a regression algorithm tree applied to 19 indeterminate FNA samples showed that the miRNAs: miR-885-5p, mir-221 and mir-574-3p enabled distinction of follicular thyroid carcinomas from hyperplastic nodules with 100% accuracy. Novel mir-885-5p is upregulated in oncocytic FTC, but not in classical FTC, adenomas and hyperplastic nodules.
Applying miRNA expression as a marker of thyroid malignancy shows promising results. However, using large scale platforms, such as microarrays, is still an expensive technology for routine laboratory use. On the other hand, quantification of miRNAs by RT-PCR is feasible and affordable in terms of cost. The only question that remains open is designing an miRNA panel suitable for a diagnostic purposes.

4. Protein-based assays

Several biomarkers have been used for the diagnosis of thyroid cancer in immunohistochemistry, alone or combined in panels. These markers belong to different categories, such as those involved in cell adhesion (E-cadherin, galectin-3, fibronectin), cell cycle regulation (cyclin D1, p27), gene transcription control (thyroid transcription factor-1), thyroid hormone synthesis and secretion (thyroid peroxidase, thyroglobulin), cellular structure (cytokeratin-19) and others. We will focus here only on the markers that are most frequently used in immunocytochemistry for FNA diagnostics.
Thyroid peroxidase (TPO) is an enzyme that helps iodination of thyroglobulin and coupling iodothyrosine to thyroxine[53]. In FNA biopsies, when immunoreactivity of TPO was absent in 80% of the cells, prediction of malignancy had sensitivity between 97% and 100%, and specificity ranging from 68% to 90%[54,55].
Antibodies to different cytokeratins have been employed in attempts to identify different expressional patterns in nonmalignant tissue, benign lesions and malignant thyroid gland tumors. CK-19 is a keratin often used keratins due to abundant expression in PTC, while it is less expressed in follicular adenomas and follicular carcinomas[56]. Some authors claim that CK-19 is a useful tool for diagnosis of papillary carcinomas in indeterminate FNA biopsies[57,58]. When CK-19 was used as a single marker, the sensitivity and specificity were 92% and 97% respectively. When it was employed in combination with Gal-3, sensitivity and specificity of nearly 100% was reached[59].
HBME-1 is a monoclonal antibody that recognizes an unknown antigen in the mesothelioma cells, normal tracheal epithelium, and adenocarcinoma of the lung, pancreas and breast[60]. Overall in the thyroid, HBME-1 stains mostly follicular-derived malignant tumors and poorly differentiated carcinomas[59]. Due to its high sensitivity and specificity, it has become

one of the most frequently used markers. In the assessment of thyroid lesions by FNA, HBME-1 has sensitivity of 80% and specificity of 96%, when used as a single marker for distinction of benign from malignant nodules[59]. Interestingly, it has been reported that in Ras mutation positive follicular tumors, the HBME-1 immunostaining was present[61].
Galectin-3 (Gal-3) is a member of the non-integrin, β-galactoside-binding lectins. It has affinity for CEA, IgE, laminin and other mucins[62]. Gal-3 is expressed by human macrophages, neutrophils, Mast cells and Langerhans cells. It is involved in the cell cycle and apoptosis, cell-cell interactions, cell-matrix interactions, adhesion and migration[62]. In the thyroid, several reports have shown upregulation of expression in malignant tumors[63-65]. Gal-3 detection by immunocytochemistry has been used in order to improve diagnostic accuracy in thyroid nodules. In a large multicenter study with 226 FNAs, Gal-3 staining resulted in 100% sensitivity and 98% specificity[66]. In a study by Saggiorato et al. on 125 thyroid aspirates, the sensitivity and specificity of Gal-3 as a single marker was 92% and 94% respectively[59]. On the other hand, a significantly lower sensitivity of 52% was reported in the smaller study of Maruta et al.[67]. Overexpression of gal-3 is suitable for distinction of malignant vs. benign lesions. Galectin-3 expression is a complementary and useful diagnostic method in indeterminate thyroid FNA samples[66].
CXCR4 is a receptor for the C-X-C ligand 12 chemokine, involved in chemotaxis, cell migration and cell adhesion[68]. When CXCR4 was used in combination with HBME-1 in preoperative cytology of indeterminate FNAB, the expression of these two markers was associated with malignancy and reached diagnostic accuracy of 90.7%[69].
The reported sensitivity and specificity of protein markers are widely variable, due to the interobserver variability in interpretation and grading of the slides. However, the results can be improved by combining protein markers with RNA or DNA-based markers.

Conclusion

FNA biopsy has an important role in the evaluation of patients with a thyroid nodule. If the FNA results are indeterminate, the patients undergo thyroidectomy. Approximately 75% of these patients have a benign diagnosis at the final histopathological examination. Molecular diagnostic becomes imperative to avoid the unnecessary surgery and to improve the preoperative risk assessment. Performing molecular analyses for somatic mutations, gene and miRNA expression, and immunocytochemistry enables patients to be separated into low-risk and high- risk categories.
Somatic mutation and genetic rearrangement tests have high specificity, but poor sensitivity in differentiating benign from malignant indeterminate thyroid lesions.
Gene expression platforms are reliable for detecting benign nodules, but are suboptimal for discovering a malignancy.
miRNA expression via RT-PCR is an achievable and promising method to evaluate indeterminate thyroid lesions, has several advantages over gene expression, but the consensus about the panel of which miRNAs are to be tested, is still to be established.

Immunocytochemistry of Gal-3, HBME-1, CK-19 and other markers have been tested in indeterminate thyroid nodules, but the range of reported accuracy is wide.
The main research goal of molecular diagnostics is finding an approach that can improve preoperative risk assessment. Molecular analysis of biopsy specimens is realistic and feasible, and the results significantly improve the diagnostic accuracy of traditional cytology. The diagnostic performance of molecular analysis in combination with cytology is proven to be superior to cytology alone.
The advantages of molecular testing are both risk and cost reduction. However, the selection of the best molecular markers and the most suitable combination of markers is still a matter of debate.

Further prospects of molecular FNA diagnosis

The use of fine needle aspiration biopsy has halved the number of patients undergoing surgery[70]. The question that remains to be answered is: which potential markers/approaches can we use in order to fill the remaining diagnostic gap?
So far, the tests that have been developed can only rule-in cancer, when a mutation or change of expression is detected. The problem is to rule-out malignancy, for those patients who are not carriers of a common mutation panel.
Molecular testing is new, the advances in the field are rapid, and clinicians must stay informed, as recommendations and guidelines for use within practice are expected to evolve. The arrival of the new ATA (American Thyroid Association) Guidelines for physicians will generate a consensus opinion of experts on this subject, and provide guidelines for the most beneficial platforms.
The development of miRNA panels is a groundbreaking improvement in the diagnostics of indeterminate thyroid nodules. Novel coming technologies, such as high-throughput sequencing for the detection of miRNA differential expression[71] and the application of small noncoding RNA in clinical cancer research[72], will further improve thyroid cancer diagnostics, and hopefully, become applicable in routine testing.

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