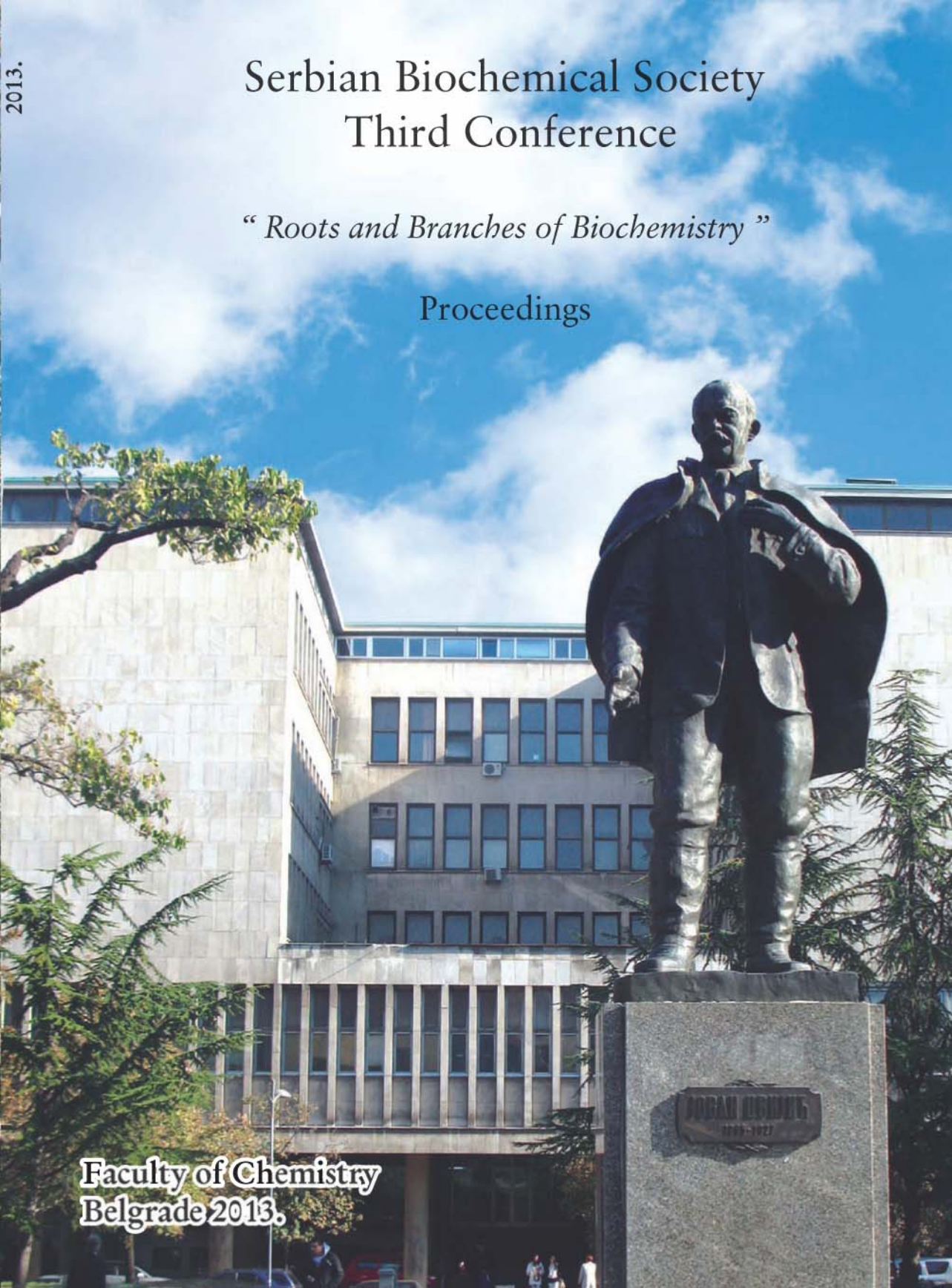
2013.

Serbian Biochemical Society
Third Conference

" Roots and Branches of Biochemistry "

Proceedings



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Serbian Biochemical Society Third Conference

University of Belgrade, Faculty of Chemistry.
8.11.2013. Belgrade, Serbia.

"Roots and Branches of Biochemistry"

PROGRAM

10.00-10.05 Welcome messages from:
Prof. M. B. Spasi}
(President of the Serbian Biochemical Society)
Prof. B Jovan~i}evi}
(Dean of the Faculty of Chemistry)

10.05-10.45 *FEBS lecture*
Prof. Mathias Sprinzl
(Chairperson of the FEBS Working Group on Integration)
Laboratorium für Biochemie, Universitti Bayreuth, Germany
Perspectives of Nucleic Acid Biosensors for medical applications

10.45-11.05 Radivoje Prodanovi}, PhD
Department of Biochemistry, Faculty of Chemistry,
University of Belgrade, Belgrade, Serbia
Flow cytometry and microfluidic lab on chip based ultrahigh throughput screening systems for directed evolution of proteins

11.05-11.30 Short break

11.30-11.50 *ESDPPP lecture*
Milica Baj~eti}, PhD
(President of European Society for Development of Perinatal and Paediatric Pharmacology)
Clinical Pharmacology Unit, University Children's Hospital, Belgrade, Serbia
Department of Pharmacology, Clinical Pharmacology and Toxicology, School of Medicine, University of Belgrade, Serbia
Efficacy, safety and quality of drugs in children

11.50-12.10 Vesna Koji}, PhD
Oncology Institute of Vojvodina, Experimental Oncology Department,
Sremska Kamenica, Serbia
Synthesis and biological activity of tiazole C-nucleosides

12.10-13.00 Short break

13.00-13.20 Perica J. Vasiljevi}, PhD

Department of Biology and Ecology, Faculty of Science and Mathematics, University of Ni{, Serbia

Potential of HAp composite scaffolds and bone marrow stem cells in bone repair

13.20-13.40 Aleksandra Zeljkovi}, PhD

Department of Medical Biochemistry, Faculty of Pharmacy, University of Belgrade, Serbia

Electrophoretic characterization of low-density and high-density lipoprotein subclasses and its significance in ischemic cardiovascular and cerebrovascular diseases

13.40-14.00 Marko N. @vanovi}, PhD

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

Electrochemical Analysis of Polyamino Acids and Proteins

14.00-14.30 Break with refreshments provided

14.30-14.50 Aleksandra Uskokovi}, PhD

Department of Molecular Biology, Institute for Biological Research "Siniša Stankovi}", University of Belgrade, Belgrade, Serbia

β–glucan-enriched extract ("Actiglucane") activates beneficial hepatic antioxidant and anti-inflammatory mechanisms in streptozotocin-induced diabetic rats

14.50-15.10 Ivona Baricevic-Jones, PhD

Institute of Cancer Sciences, University of Manchester, Manchester, UK

Signalling and mitogenic activity of insulin analogue X10

15.10- Discussion and concluding remarks

Foreword

Dear Colleagues,

It is my great pleasure to wish you warm welcome to the Third Conference Entitled “Roots and Branches of Biochemistry” organized by the Serbian Biochemical Society.

Official language for Third Conference of the Serbian Biochemical Society is English. We have invited President of FEBS Working group for integration Mathias Sprinzl to be lecturer and seven from Serbia to present their state of art in the field they work as invitation for further co-operation. This year one member of Serbian Biochemical Society with affiliation from abroad research institution is also invited speaker as beginning of cooperation with Diaspora. Their presentations are published in Proceedings.

I express my gratitude to the members of governing board of Serbian Biochemical Society who suggested lecturers and to all of them who accepted Invitation.

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

Perspectives of Nucleic Acid Biosensors for medical applications; Electrically readable biochips for rapid RNA analysis

Mathias Sprinzl *

* *Laboratorium für Biochemie, Universität Bayreuth, Germany*

Transduction of biochemical events arising from nucleic acid hybridisation to electrically readable signals is the objective of the presented research. The aim is to develop analytical devices (biochips) for use in diagnostics, biotechnology and environmental analysis.

In our approach, nucleic acid hybridisation directs a thermostable esterase[1] for binding to gold electrodes where an electrochemically detectable p-aminophenol is enzymatically synthesized and detected as a redox reaction – dependent current.

All reactions are performed directly on CMOS chips presenting 128 electrodes in a volume of about ten microliters, or less. Technical solutions and optimized biochemical procedures improving the sensitivity and specificity will be discussed. Applications for detection of bacteria and microRNA will be presented[2,3].

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Proceedings

Flow cytometry and microfluidic lab on chip based ultrahigh throughput screening systems for directed evolution of proteins

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Directed evolution is a powerful method that involves iterative rounds of diversity generation and diversity screening for improved protein variants. It enables us to evolve proteins with desired properties and to study protein evolution and protein structure-activity relationships. The most limiting step of directed evolution is a screening process since with diversity generation methods we can create up to 10^{12} different protein variants. In order to overcome these limitations recently *in vitro* compartmentalization methods combined with flow cytometry and microfluidic lab on chip technology were combined and ultrahigh-throughput screening methods for thiolactonase, galactosidase, proteases, peroxidase, glucose oxidase, and cellulases were developed.

Introduction

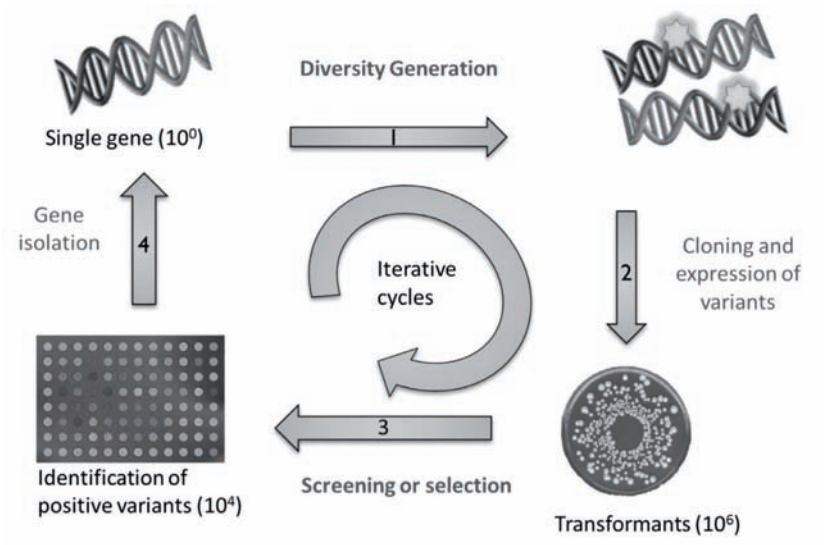
Proteins are macromolecules designed by nature to perform various biochemical reactions and processes in living organisms. Protein 3D structure that determines activity is governed by protein primary structure (amino acid sequence) and it is optimized by evolution process that occurs in nature to suit its function in a living organism[1].

Directed evolution of proteins

If we want to use a specific protein for human needs we will encounter a problem of compromised activity and stability in operating conditions that are usually very different from *in vivo* conditions for which protein was evolved by nature[2]. Therefore there is enormous need for optimizing protein performance in a changed environment required by industrial processes where protein is applied. One of the approaches to achieve this goal is protein engineering, where molecular biology techniques are used to change protein structure on a gene level[3]. Rational protein design requires knowledge of protein structure and understanding of structure-function relationships. Since we are still far away from understanding how structure influences protein activity guided by the nature scientists are

using directed evolution as a tool for protein design. Directed evolution uses principles of Darwinian evolution from nature and applies it in the lab[4]. In an iterative process of mutation/recombination and selection, proteins are forced to evolve in the direction needed for better performance in industrial operation conditions, Scheme 1.

Directed evolution is also a tool for studying protein evolution and understanding protein structure-function relationship[5]. Since using this methodology we are working with big numbers and gene libraries with sized of up to the 10^{12} different protein mutants the most limiting step is a selection/screening process[6]. There is an enormous effort today to develop high throughput screening systems (HTS) for gene libraries that will enable us to measure protein activity of a very high number of variants in a very small time frame using small amount of expensive reagents.

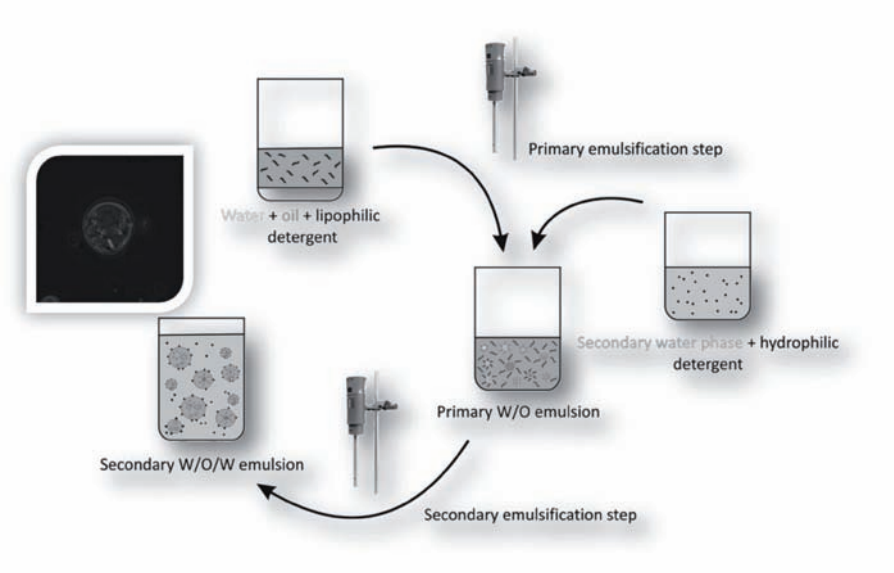


Scheme 1. Directed evolution of proteins.

High-throughput Screening Systems and *in vitro* compartmentalization

The most of high throughput screening systems are performed inside the wells of microtiter plates (MTP). Using MTPs where reaction volumes are in the range from several microliters to several hundreds of microliter we can screen gene libraries with a sizes of up to 10^5 in a reasonable time frame from several days to several months depending on a specific screening system and use of robotic systems. In order to overcome this limit recently new

technology named *in vitro* compartmentalization (IVC) was suggested. Guided by the nature and evolution process Tawfik and Griffith[7] designed screening systems that are performed in water microdroplets of water-in-oil-in-water emulsions, Scheme 2.

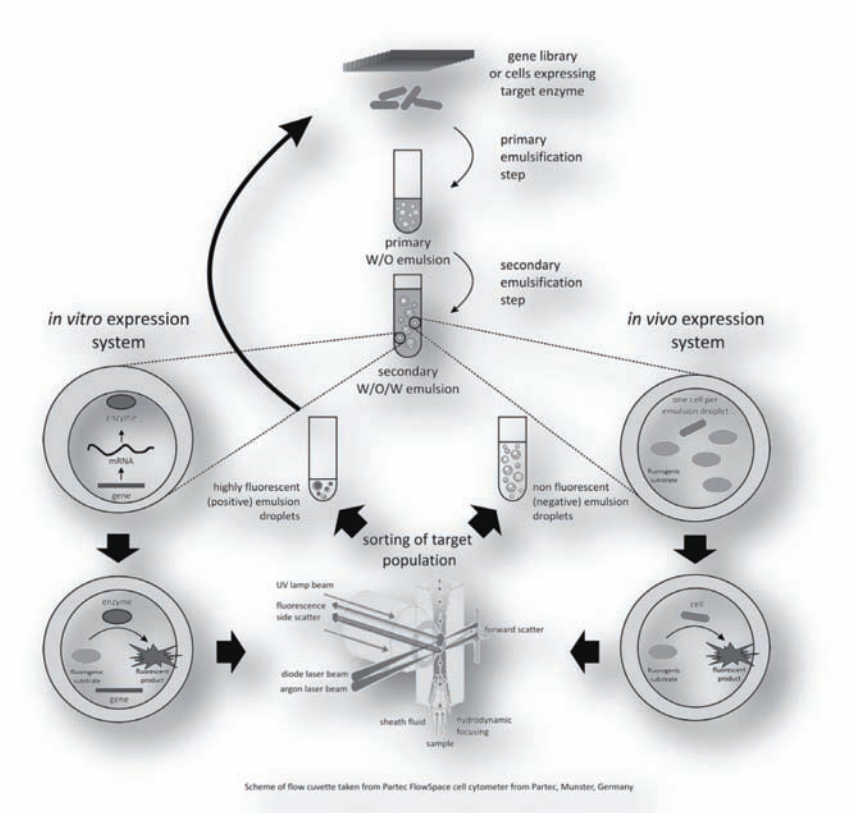


Scheme 2. Compartmentalization of gene libraries inside double emulsion of water-in-oil-in-water (W/O/W). *E.coli* cells inside one double emulsion droplet are shown with a green fluorescence on a real experiment microscope image pasted on the scheme.

The diameter of these water microdroplets ranges from 0.5 to 10 μm and we can have 10^{10} reaction compartments in 1mL of emulsion. Using this approach we can perform huge number of assays in a very small volume. In order to measure a signal coming from these microdroplets we have to use either selection screening system based on affinity/binding[8] or screening system based on fluorescence and modern devices such are flow cytometers[9,10] or microfluidic lab on chip devices[11].

Flow cytometry based screening systems

Flow cytometer or FACS (fluorescence activated cell sorter) is a device that uses a laser beams to excite reaction microcompartments surrounded by a water sheet fluid, measure fluorescent signal coming afterwards and sort them at a very high speed of up to 10^4 particles per second according to fluorescence, particle size and shape, Scheme 3.

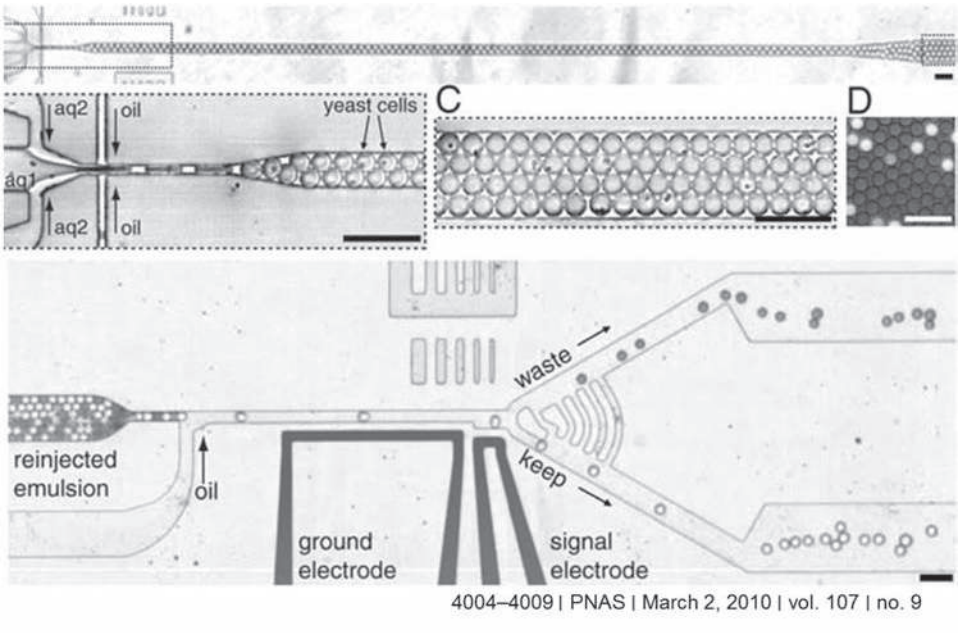


Scheme 3. Flow cytometry screening process of gene libraries expressed inside cells or using *in vitro* translation systems.

Using this approach screening systems for directed evolution of thiolactonase[9], galactosidase[10], glucose oxidase[12], protease, cytochrome P450, and cellulase were developed that enabled screening of gene libraries with sizes of 10^7. For example ultrahigh throughput screening system for glucose oxidase enabled screening of 10^7 different enzyme variants in less than half an hour that resulted in finding improved glucose oxidase variants[12]. Flow cytometers require for sorting a water sheet fluid so that only suspension of cells in water[12] or double emulsions can be sorted[13]. There are however drawbacks of screening technology based on double emulsions: high complexity and polydispesity of emulsion structure that is limiting quantitative analysis and difficulty to modify content of droplets after gene/cell compartmentalization. These limitations can be overcame by using droplet-based microfluidic systems that allow generation of highly monodisperse single emulsions (water-in-oil), and fusion and splitting of droplets.

Microfluidic lab on chip devices

Recently, drop-based microfluidic device was used for development of an ultrahigh-throughput screening platform that enabled screening of 10^8 individual enzyme reactions in only 10 h[11]. Using this device horseradish peroxidase expressed on the surface of *Saccharomyces cerevisiae* EBY100 cells was evolved to catalyze 10 times faster oxidation of a fluorescent dye Amplex Red, Scheme 4.



Scheme 4. Photographs of microfluidic lab on chip device used for directed evolution of horse radish peroxidase and later on glucose oxidase.

The major challenge for IVC technology remains development of fluorescent assays that will reflect industrially relevant activity we are aiming to evolve, and that will fit to the complex (bio)chemical environment of a single cell protein activity measurement in water microdroplets. One example of such an assay is vanadium bromoperoxidase coupled assay for glucose oxidase[13] that can be used for directed evolution of glucose oxidase activity relevant for industry and can be easily adapted for other hydrogen peroxide producing enzymes or for other glycosidases like it was in the case of cellulase[14]. ViPer assay for glucose oxidase and cellulase previously developed for use on a flow cytometer and IVC technology in double emulsions was successfully adapted for use on a microfluidic lab on chip devices described here in single emulsions.

Conclusion

Directed evolution is a powerful method for studying protein evolution, structure-function relationships and creating proteins with superior properties compared to their parents (wild type forms). In order to have successful experiment during directed evolution it is necessary to screen huge number of protein variants and therefore screening process is the most limiting step. The most promising ultrahigh throughput screening systems are the ones based on *in vitro* compartmentalization of genes encoding proteins inside microcompartments that will be after performing protein activity assay sorted using flow cytometers or lab on chip microfluidic devices. Further development of screening technology requires improvement of microdroplet handling using such devices. At the end at the heart of each screening process is the protein activity assay that should fit to the requirements of used devices, physic chemical environment of the microreaction compartment and protein property that we want to evolve.

Acknowledgements

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Efficacy, safety and quality of drugs in children

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The development of medicines for children is one of the main challenges in modern clinical pharmacology. Key issues are drug evaluation for paediatric use, age-appropriate formulations, safety of exipicients, suitability of drug delivery devices and therapy compliance. In conjugation with the knowledge about efficacy, pharmacokinetics and pharmacodynamics the impact on the objective of making available safe medicines for children will steadily improve. Currently, one of the limiting factors for access to medicines in the paediatric population is the availability of licensed formulations that suits the age of the child. Treating neonates, infants, and children with off label and "homemade" products is regarded as unacceptable, as the efficacy and safety have not been tested for the intended indication, disease state, nor the developmental stage of the child.

Introduction

A safe and effective medication for children requires a fundamental understanding and integration of the role of ontogeny in the disposition and actions of medicines. The father of American paediatrics, Abraham Jacobi (1830-1919) recognized the importance of need for age appropriate pharmacology when he emphasized "*Paediatrics does not deal with miniature men and women, with reduced doses and the same class of disease in smaller bodies*". Unfortunately, this fundamental knowledge of determining the paediatric dosage of a medicine is even today, 100 years later, often ignored.

Rational use of drugs in paediatrics requires specific knowledge[1]. The infantile organism cannot be observed as a diminished adult organism, because the physiological treats of child's organism differ from the organism of a healthy adult[2]. From the safety aspect, neonates and children form particular group of patients, so called risk or vulnerable group. They differ from adults in physiological, psychological and developmental sense. Growth and development are dynamic processes. Growth is a continuous change of mass, shape, proportions and physiological functions during ontogenesis, whilst development consists of qualitative changes during biological maturation – changes in the function of cells, tissues and organs,

enzyme induction, reorganization of regulatory mechanisms. With the aim of improvement of drug use in children, it is important to possess knowledge of complex processes of growth and development due to their influence on pharmacokinetics and pharmacodynamics of a drug[3]. In addition, it is necessary to pay attention to the specifics of diseases in children, influence of the environment and hereditary factors. For example, the aetiologies of heart failure (HF) in children are different and more diverse than in the adult population[4]. In children, HF is most often caused by congenital heart disease (CHD) and cardiomyopathy, whilst in adults the most common causes are coronary artery disease, long-time cardiac stress due to hypertension, and acquired valvular heart defects, arrhythmias and myocardial infarction. Therefore, development of designer drugs, based on HF pathophysiological knowledge in heterogeneous paediatric population at a genetic, molecular and cellular level will be of great importance[5]. Additionally, the lack of pharmacokinetic and pharmacodynamic data for drugs used in children increases the risk of overdose and subdose, adverse affects and inefficacy of a generally efficient drug[6]. Therefore, drugs evaluation for paediatric use should be performed in each stage of children development in order to achieve adequate safety and efficacy level. The paediatric population extends from the preterm and term newborn infant through childhood, and adolescence, or even to young adulthood. The internationally agreed and to some extent arbitrary, classification of the paediatric population is as follows:

- Preterm infants (<37 week gestation)
- Term newborn infant (0-28 days)
- Infant and toddler (>28days to 23 months)
- Children (2-11years)
- Adolescents (12 to16-18 years)

Off-label and unlicensed drug use in children

The drug license refers to the assessed and confirmed efficacy, safety and quality of the drug. Precisely defined drug instructions in the Summary of Product Characteristics (SmPC) and Patient Information Leaflets (PIL) are the result of years of preclinical and clinical assessments. Unlike drugs used in the management of disease in adults, only a small proportion of drugs are licensed for use in children[7,8]. Due to law prohibition on performing clinical study in children, the majority of drugs aimed at therapy in children are prescribed unlicensed or *off-label*, which means that the therapy is not based on evidence, but rather on sense, experience, and extrapolation. Cardiovascular drugs are - apart from analgesics and antibiotics - the most prescribed *off-label* and unlicensed drugs in children[7-10]. The common denominator of this phenomenon is the lack of adequate clinical studies.

A survey on unlicensed and off label use in paediatric wards in European countries has shown that as much as 46% of drug prescriptions were either unlicensed (39%) or off label (7%), and that 67% of patients received an unlicensed or off label drug prescription[10]. Our study first pointed out the anomaly of the lack of paediatrically designed and evaluated drugs for the treatment of heart failure (HF)[7]. During a two-year review of drug prescriptions at the paediatric cardiology and cardiosurgery ward of the University Children's Hospital

in Belgrade, it was found that the majority of unlicensed and *off-label* drugs consisted of drugs aimed at the cardiovascular (CV) system. Of the total number of CV prescriptions, more than half were unlicensed (9%) or *off-label* (44%). 76% of the children received at least one or more *off-label* or unlicensed drug. The highest percentage of unlicensed drug use was recorded in children aged 2-12 years (17%), whilst the highest percentage of *off-label* drugs was prescribed to the neonates (64%). It is interesting to note that the *off-label* drugs that are most often prescribed due to inadequate dose are the first-choice drugs in HF treatment: furosemide, digoxin, spironolactone and captopril. Even the paediatrically evaluated drugs were prescribed *off-label*. Up to one third of captopril prescriptions were *off-label* due to excessive dose. There was also a tendency to prescribe excessive doses of furosemide (25% of prescriptions) and spironolactone, especially in children with severe HF. Pasquali *et al*., found that 6 CV drugs - enalapril, sotalol, lisinopril, amlodipine, losartan and fenoldopam - approved for children in the meantime, were still being prescribed *off-label* (62.3% of the time)[8]. In research conducted on 31,442 paediatric patients in the USA, who were undergoing treatment for congenital HF and other cardiac diseases, it was shown that *off-label* use of CV medications is common in children hospitalized with congenital or acquired CV diseases, with 78% receiving one or more *off-label* drugs. Heart transplant patients and patients undergoing congenital heart surgery received the greatest number of *off-label* CV medications. They also found that neonates were more likely to receive a greater number of *off-label* CV medicines[8].

Even though there is no sufficient data addressing the association between the usage of *off-label* drugs and adverse reactions, it is certain that such drug use can significantly jeopardize the efficacy, safety and quality of treatment in children[11]. For instance, recently published studies showed that supplementation of vitamin E significantly increases the risk of infection and sepsis despite the current recommendation that preterm infants should be routinely supplied with vitamin E[12,13]. The recently published study by Aagaard and Hansen shows that *off-label* prescribing of drugs in Denmark from 1998 to 2007 was associated with a large number (50%) of serious adverse drug reactions (ADRs), including fatalities[14]. More than half of *off-label* ADRs were reported in adolescents. This evidence highlights the need for systematic drug research in relation to the child population.

Lack of drug formulations labelled for treatment in children

Despite the growing number of commercially-produced formulations for adult treatment, most drugs are not suitable for neonates and young children[7,8]. Drugs are mostly manufactured in formulations that meet the needs of adult patients requiring fixed doses. Neonates and young children require suitable oral (*e.g.* liquid, *etc.*) and intravenous formulations, as they are unable to swallow tablets and capsules, and also need doses based on body weight[15]. Intravenous drugs marketed for adults are often too concentrated for the accurate measurement of small doses for neonates and young children. For example, injectable digoxin is available at 500 micrograms per 2 ml. Measurement of small volumes to provide daily maintenance doses in neonates, from 4 to 8 micrograms per kilogram, can be associated

with inaccurate administration and intoxication[16]. In order to provide proper administration of drugs, medications must be available in age-appropriate formulations in order to achieve the desired therapeutic outcome and avoid adverse effects[15]. Moreover, recent reports on paediatric drug trials involving younger children have documented the lack of inadequate information provided on the precise oral formulations being investigated, thus reducing the validity of the published paediatric trials[17].

The lack of age-appropriate oral formulations for children is a global phenomenon[18-22]. Our survey shows that in 2007, in all 4 investigated markets (USA, Germany, Norway and Serbia), there was a total absence of labelled antihypertensive drugs in oral formulations suitable for young children[18]. The USA had the highest percentage of labelled, child-size oral dosage forms (47.7%), followed by Germany (39.1%), Norway (26.8%), and Serbia (21.2%). Anti-infective agents and drugs to treat respiratory diseases had a high availability in the USA and Germany (threshold >50%). In Serbia and Norway, the threshold of more than 50% availability was not reached in any anatomic drug class. In all four countries, therapeutic drug classes with a total absence of oral formulations were anti-obesity drugs, anti-hypertensives, sex hormones, and antineoplastic agents[18].

The lack of commercial drugs suitable for children is a big safety problem. A large number of CV drugs are prepared in local pharmacies from tablets and capsules, whilst injection solutions are diluted; as a result, the bioavailability and quality of these preparations is highly unreliable. A survey by Mulla *et al.* showed that paediatric cardiac centres and referring hospitals in the UK use, interchangeably, a variety of unlicensed captopril formulations in order to treat children with HF[23]. In relation to captopril and paediatric HF, no bioequivalence data exist for the liquid formulations identified in the survey. This degree of inconsistency raises issues about optimal captopril dosing and potential toxicity, to the point that its use may influence paediatric cardiac surgical and interventional outcomes[23]. Also, due to the lack of commercial drugs for oral use in children, it often happens that drugs for parenteral application in adults are used as oral preparations in sick children (*off-label* with regard to the route of administration), which exposes them to a higher risk of the drug's adverse effects[10].

The splitting of tablets can lead to the erroneous dosing of a drug or may cause toxicity (e.g. film tablets); grinding of tablets can impact drug absorption, which can result in therapeutic failure[15,24]. Improvised preparation of liquid drug formulations in local pharmacies, *i.e.*, the chopping of tablets or opening of capsules for adult use, contributes to the risk of inefficiency and the unpredictable safety of the therapy. Such modified preparations are marked as unlicensed drugs, if modification of a commercial preparation is not tested. The study that assessed the bioequivalence of two liquid unlicensed captopril preparations against a licensed tablet form shows that they are not bioequivalent to each other and thus cannot be assumed to behave similarly in therapeutic use[25]. There is also a significant variability in within-subject performance, which has clinical implications with respect to titrating to an optimum therapeutic dose[25].

For modified preparations administered intravenously it is necessary to test their sterility and pyrogenicity. Obligatory tests for oral drug use are as follows: potency of active substance, bioavailability, and stability under normal circumstances[15]. Unfortunately, in practice, data regarding the bioavailability, efficiency and safety of extemporaneously prepared drugs are often unavailable. Santovena *et al.* studied five different hydrochlorothiazide oral formulations prepared with traditional compounding techniques in pharmacies in order to treat HF and oedema in babies[26]. There was only one studied formulation that guarantees the correct dose administration and stability after 3 weeks storage at 5°C, in a light-protected atmosphere.

Besides that, the excipient, the adjunct substance used in the extemporaneous preparation of a drug - generally a pharmacologically inactive substance - can incite adverse effects in children. It is indicated in literature that, in young children, propylene glycol can cause hyperosmolality; sweeteners can increase sugar levels in blood; sorbitol and xylitol can cause osmotic diarrhoea; lactose causes intolerance, *etc.*[27,28]. Unlike active substances, excipients are not well regulated in most countries. An on-going study, entitled "A prevalence survey of neonatal excipients exposure in 27 EU countries, Serbia Switzerland, Norway and Iceland (ESNEE Point prevalence study)", will provide information about individual excipient-exposure in neonates and highlight the needs and opportunities for product substitution and priorities for reformulation[29].

A liquid dosage form is necessary in order to accurately measure doses according to the child's body weight[15,24]. Development of drug formulations for alternative routes of administration, *e.g.* through gastric tube, nasal, pulmonary, and transdermal, is an important step to avoid manipulating tablets of adult dosages[30]. The introduction of fast-dispersing tablets and other novel formulations should be encouraged, since they facilitate the administration of low doses. In order to improve the situation, a number of international initiatives has been established. Amongst others, the World Health Organization has launched a global campaign: "Make medicines child size", while the European Paediatric Formulation Initiative (EuPFI), a group consisting of paediatric formulation experts from industry, academia, and clinical pharmacy was founded with the aim of raising awareness of paediatric formulation issues[30].

Regulation and paediatric HF drug development

In spite of numerous incentives in relation to the stimulation of paediatric drug research, children still do not have efficient, safe and quality treatment, as is available to adults. The Paediatric Regulation No 1901/2006 entered into force in Europe on 26 January 2007, with the aim of improving the development of medicinal products, addressing the lack of age-appropriate formulations and providing information on efficacy, safety and dosing for the paediatric population[31]. The Regulation requires applications for marketing authorisations to be accompanied by either a product-specific waiver or a paediatric investigation plan, to be agreed upon by the Paediatric Committee (PDCO) of the European Medicines Agency (EMA). In the EU, from 2007, every new drug under development must have a paediatric

investigation plan (PIP)[32]. The European network of paediatric research (EnprEMA) was established by EMA in 2010[33]. The establishment of a network of paediatric research networks, giving public access to paediatric trials included in the EU Drug Regulating Authorities Clinical Trials database (EudraCT), and the funding of research on off-patent medicines prioritised by PDCO, was a measure begun by the Regulation[34].

In the USA, ten years before the EU, the Paediatric Exclusivity Provision provided incentives for conducting safety and efficacy studies in children[35]. The Best Pharmaceutical Act of 2002 created a mechanism to study frequently-used older drugs with expired patents[36]. For example, the Paediatric Heart Network, a network boasting 8 clinical centres specially designed to develop and apply an evidence-based approach to paediatric CV disease, was established by The National Heart, Lung and Blood Institute in 2001[37]. Since it was established, the PHN has initiated several trials related to HF, the benefits of which are already being explored.

Through a stringent system of obligations compensated by economic rewards, a dramatic change in the way medicines are developed is taking place. These new measures are implemented within an ethical framework to obtain evidence–based information while protecting the children involved in research.

Conclusion

In order to reach the current standard of adult pharmacotherapy, there is an urgent need for clinical research and development of drugs for children. The peculiarities of the different and more diverse disease aetiology, in addition to major differences in the pharmacokinetics/pharmacodynamics of drugs in children, dictate that paediatric management should be approached with a different perspective than is applied to adults. Development of designer drugs based on pathophysiological knowledge as well as drug formulations suitable for children are basic steps that are necessary for the efficacy and safety of therapy in children.

Acknowledgements

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Synthesis and biological activity of tiazole C-nucleosides

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A total synthesis of new acyclic tiazole C-nucleosides bearing a double bond or 2′,3′-dideoxy functionality in the sugar moiety was achieved in this work. The multi-step synthesis of the tiazofurin analogues was based on D-arabinose as a chiral precursor. In vitro cytotoxic activity of newly synthesized compounds was evaluated against selected human leukemia, lymphoma and solid tumor cell lines. A study of cell mechanisms underlying the significant cytotoxic potential of these molecules was carried out and the results were compared to tiazofurin that served as a referent compound. Depending on compound configuration and cell exposure time, the investigated analogues expressed different anti-proliferative activity. The changes in the cell cycle phases of K562 cells were compound- and time-dependent. Synthesized tiazofurin analogs induced time-dependent apoptosis in K562 cell line, which was greater, compared to tiazofurin.

Introduction

The necessity to develop more efficient and less toxic antitumor drugs induced investigations towards new pharmacologically active substances. This necessity was particularly important for the majority of common cancers such as cervix, breast, lung and colon cancers. Presently, chemotherapy and hormonal drugs are not efficient enough due to the unspecific mechanisms of action[1]. Besides, the resistance of many tumors to existing therapies still represents one of the main problems in the treatment of malignant diseases. Thus, the strategy in development of new drugs based on overcoming the tumor drug resistance should contribute to longer survival of cancer patients[2]. Most of antitumor treatments, including chemotherapy, radiation, and immunotherapy or cytokine therapy act primarily by induction of apoptosis in tumor cells. Defects in the apoptotic process may lead to drug resistence[3].

The aim of this work was evaluation of molecular mechanisms underlying the antitumor activity of newly synthesized tiazofurin analogues. We used a panel of cell lines - nine malignant and one normal cell line, for evaluation of antiproliferative activity of new tiazofurin analogues. We showed that tiazofurin derivatives with modified carbohydrate segment exerted a stronger anti-proliferative activity against some cell lines then the tiazofurin itself. Our experiments clearly showed that the presence of ribofuranosil moiety was not necessary for the antitumor activity of tiazofurin as it was previously stated.

Synthesis of tiazofurin derivatives

Several natural and synthetic C-nucleosides have been already included in chemotherapy regimes as antitumor, antivirus and anti-bacterial agents. They also act as immune modulators and regulators of gene expression[4, 5]. Tiazofurin (2-β–D-ribofuranosiltiazole-4-carbixamide) is the first synthetic C-nucleoside with significant antitumor activity[6], which is registered as "orphan drug" for chronic myeloid leukemia (CML) therapy in blast crisis. Biological activity of tiazofurin is a result of its ability to reduce the intracellular depot of guanine nucleotides by the inhibition of IMPDH (inosine monophosphate dehydrogenase).

Synthesis of more specific and effective inhibitors of IMPDH could make progress in the treatment of diseases, which have increased activity of this enzyme.

Searching for such IMPDH inhibitors, which are more selective and more effective then tiazofurin, resulted with a synthesis of two new tiazole acyclic C-nucleosides with a double bond and 2',3'-dideoxy function in acyclic sugar component based on D-arabinose as a chiral precursor.

Initial stage of our research involves multi-phase synthesis of acyclic tiazofurin analogues **9** and **12**. The synthesis starts from diethylditioacetal D-arabinose 2, and by further chemical changes of substituents, free C-nucleosides **9** and **12** were obtained (Figure 2., Figure 3.)[7].

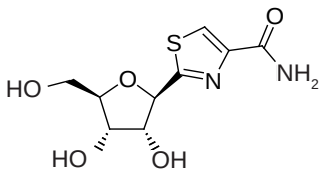
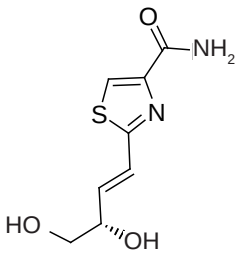


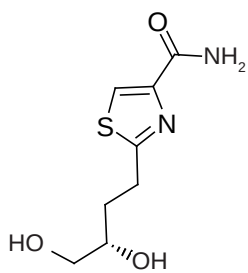
Figure 1. Tiazofurin

Acyclic tiazole C-nucleosides



9, (E), (S) -2 - (3,4-dihydroxy-but-1-enyl) thiazole-4-carboxamide

Figure 2.



12, (S) -2 - (3,4-dihydroxy-butyl) thiazole-4-carboxamide

Figure 3.

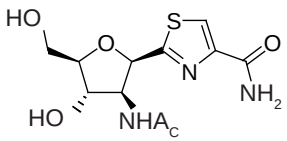
Investigation of anti-proliferative activity

Successful completion of development more effective anticancer agents involves searching for compounds which would be able to induce stronger growth inhibition of neoplastic cells at low concentrations.

We investigated in vitro cytotoxicity of newly synthesized acyclic analogues (**9** and **12**) and three analogues having arabinose configuration (**13**-**15**) (Figure 4-6.)[7-9] against several cell lines of human leukemia and solid tumors, and tiazofurin served as a reference compound. Anti-proliferative activity was examined by the MTT[10] assay, on selected human leukemia cell lines (K562, HL-60, and Jurkat), Burkitt's lymphoma (Raji), solid human tumors (HT-29, MCF-7, MDA-MB-231 and HeLa) and the cells of normal human lung fibroblasts (MRC-5). Cytotoxicity was expressed as the IC50 value.

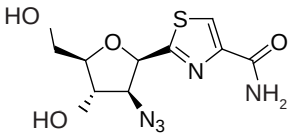
The obtained results showed a large variation in activity of analogs depending on the cell line. This indicated that newly synthesized analogues do not act on the same biological target (IMPDH) as tiazofurin. If the tested compounds affected the same biological target, analogues should exhibit similar anti-proliferative effects as tiazofurin against all cell lines. The results of cytotoxic activity tests gave us information about reduced number of viable cells in a population of treated cells compared with untreated ones. Based only on the IC50 values it was not possible to determine whether the observed anti-proliferative activity was a consequence of cell death or inhibition of cell division. Given that apoptosis is one of the key cellular pathways of chemotherapeutic activity[3,11,12], we further studied the cellular mechanisms, which underlie the observed cytotoxic potential of the synthesized analogues.

Analogues *arabino* configuration



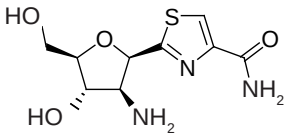
13, 2-(2-acetamide-2-deoxy-β-D-arabonofuranosyl) thiazole-4-carboxamide[8]

Figure 4.



14, 2-(2-Azido-2-deoxy-β-D-arabonofuranozil)thiazole-4-carboxamide[9]

Figure 5.



15, 2 - (2-Amino-2-deoxy-β-D-arabonofuranozil)thiazole-4- carboxamide[9]

Figure 6.

Impact of tiazofurin analogues on cell cycle distribution

We showed that the synthesized tiazofurin analogues induced changes in cell cycle distribution of K562 cells. Treatment of K562 cells with synthesized analogues for 24 h leads to accumulation of cells in the S phase of the cell cycle. During longer incubation time (72 h), both analogues (**9**, **12**, **13**, **14** and **15**) and tiazofurin caused the accumulation of cells in G0/G1 phase of the cell cycle, which was slightly less compared to the control. All analogues with *arabinose* configuration and tiazofurin, after 72-hour treatment of K562 cells, showed a significant percentage of apoptotic cells (sub-G1 peak) (Figure 7).

We also showed that the newly synthesized analogues of tiazofurin induced time-dependent apoptosis in the K562 cell line. One can speculate that the apoptotic activity of newly synthesized compounds was due to structural changes and that particular change in the chemical structure of analogue significantly yielded to its antitumor activity.

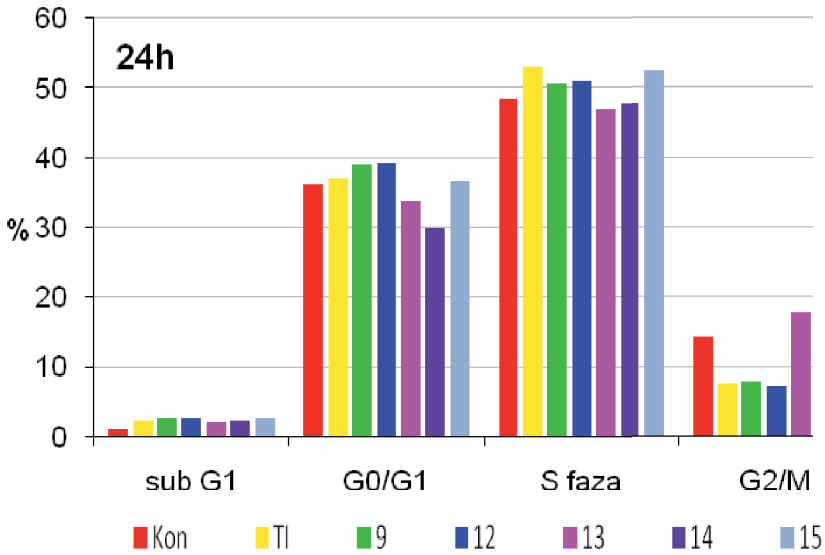


Figure 7. The impact of tiazofurin analogues on the cell cycle of K562 cells by flow cytometry; acyclic tiazole C-nucleosides (**9, 12**), analogues *arabino* configuration (**13, 14, 15**)

Apoptosis as the predominant type of cell death

Flow cytometry analysis of the cell cycle (propidium iodide staining), showed a pro-apoptotic effect of examined analogues on K562 cell line, through the establishment of a clear subG1 peak, which corresponded to the accumulated hypo-diploid apoptotic cells.
Further, we examined the type of cell death induced by the analogs on K562 cells. Using

double staining of cells with Annexin V-FITC and propidium iodide, it is possible to detect cells in the early phase of apoptotic cell death.

Investigating the cell death kinetics of K562 cells treated with tiazofurin and analogues, we found that longer exposure of cells to tiazofurin led to a slight increase of Annexin V-positive cells. That indicated that tiazofurin, after 24 h at IC50 concentrations, acted mainly as an inhibitor of Inosine-5′-monophosphate dehydrogenase (IMPDH). Apoptotic response, presented as a percentage of specific apoptosis, showed that all tiazofurin analogues after 24h and 72h increased several-fold the percentages of Annexin V positive K562 cells, compared to tiazofurin. It could be speculated that the apoptotic activity of newly synthesized compounds was actually due to their structural changes (Figure 8).

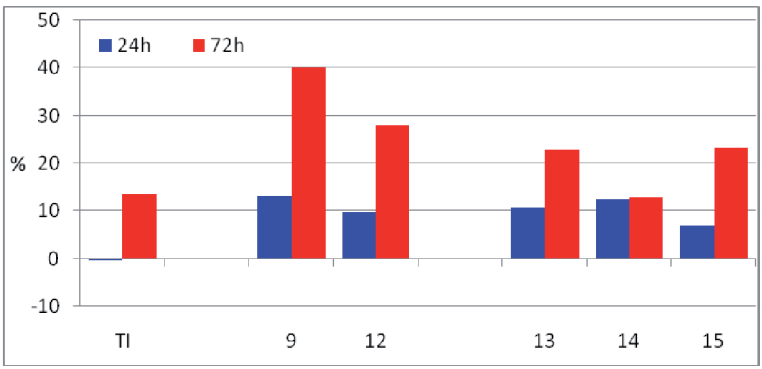


Figure 8. Percentage of specific apoptosis of K562 cells induced by tiazofurin and analogue 9, 12 -15 after 24 and 72 h-treatment. Cells were stained with Annexin-V-FLUOS and propidium iodide and analyzed by flow cytometry. Percentage of specific apoptosis of tiazofurin and tiazofurin analogues was calculated according to reference.[19]

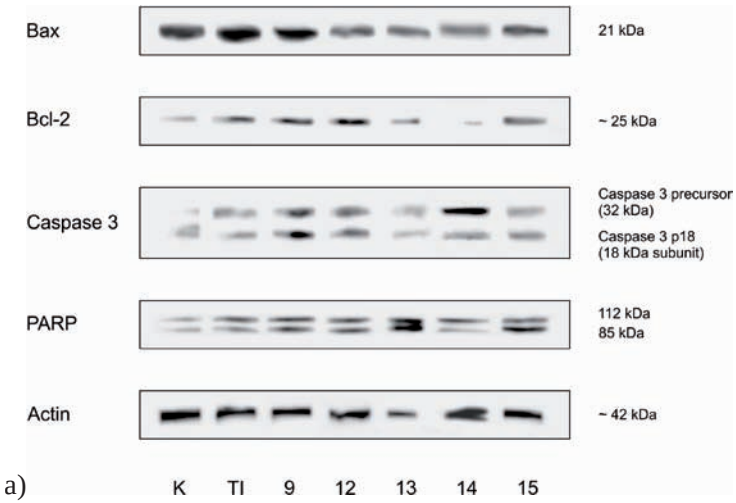
Effect of tiazofurin analogues on cell signaling molecules involved in apoptotic cascade

In order to define the antitumor activity of the synthesized analogs at the molecular level, and to detect molecular signaling pathways through which it was manifested, we investigated the influence of new analogues on the expression of „upstream“ and „downstream“ proteins in the signaling cascade of cell proliferation, differentiation and apoptosis. The effect of these compounds on expression of pro- and anti-apoptotic proteins in K562 cells was determined by semi-quantitative Western Blot method.

Western blot analysis of analogs **9** and **12-15** showed that 24-hour treatment by analogues **12** and **14** reduced the expression of Bcl-2 compared to untreated cells and tiazofurin. However, analogues **9**, **13** and **15** significantly increased the overall amount of Bcl-2 protein compared

to tiazofurin. After 72 h, analog **14** decreased the expression of anti-apoptotic Bcl-2 protein compared to control and tiazofurin, contrary to analogue **13**. Compared to tiazofurin, the expression of Bcl-2 was about the same, except for analogs **13** and **14** where the expression was lower.

Compound **14** showed the greatest decrease of Bcl-2 protein expression at both investigated times. Various chemotherapeutics induced apoptosis manifested with significant expression of Bax protein. However, in our experiments tiazofurin analogs **9**, **12-15** did not significantly affect the expression of Bax after 24 h. It was also confirmed that apoptosis in various human solid tumors could be induced in the absence of any change in Bax protein level[13,14]. The expression of Bax was increased after 72 h of treatment with all tested substances and with



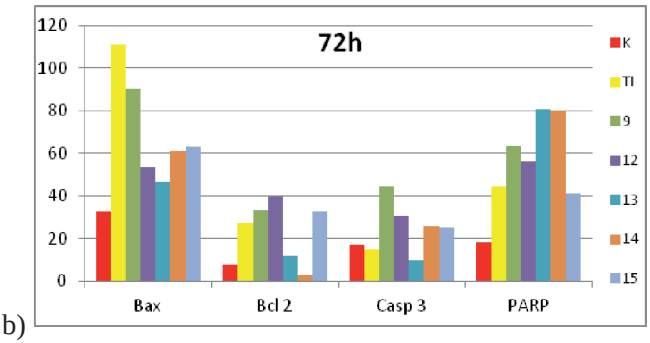


Figure 9 a, b: Western blot analysis of the protein expression of Bcl-2, Bax, caspase-3, and PARP after 72 h-treatment: control (K), tiazofurin (TI), analogues **9**, **12-15.**

tiazofurin, related to the 24 h treatment time. These results are consistent with an increase in specific apoptosis after 72 h.

Expression levels of precursor and active subunit of Caspase 3 were measured in order to determine whether these compounds induce apoptosis by activation of Caspase 3[15-18]. Treatment of K562 cells with all analogs (**9**, **12**-**15**) led to the expression of Caspase 3 protein precursor. After 24 h, all analogues except **14** and tiazofurin increased the expression of Caspase 3, which clearly indicated the involvement of Caspase 3 in the apoptotic process of treated cells. Smaller effect on the cellular content of Caspase 3 was also observed after 72-hour cell treatment with tested substances except **14** (Figure 9).

We also demonstrated the proteolytic cleaving of PARP in K562 cells after treatment with all tested analogues **9**, **12**-**15**, which depended on both the analogue's structure and exposure time.

Conclusion

Our results showed, for the first time, the significant antitumor activity of newly synthesized tiazofurin analogues against selected human tumor cell lines. Tiazofurin analogues induced the changes in the cell cycle distribution of K562 cells in compound- and time- dependent manner; they also induced higher and time-dependent apoptosis in K562 cell line compared to tiazofurin.

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Potential of HAp composite scaffolds and bone marrow stem cells in bone repair

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Abstracts

Bone tissue reconstruction and reparation is big challenge in medicine. Biocomposite materials based on hidroxyapatite are widely used in reparation of bone defects. Bone marrow is a complex tissue composed of hematopoietic and stromal mesenchyme stem cell (BMSCs) with a differentiation potential to adipogenic, fibroblastic, reticular, osteogenic and chondrogenic lineages. The aim of this study was to investigate *in vitro* and *in vivo* interaction between bone marrow cells and biocomposites based on HAp/PLLA. Changes in cell morphology with suppress cell proliferation suggest that presence of biocomposites HAp/PLLA can induce differentiation of bone marrow cells *in vitro*. In HAp/PLLA implants are visible angiogenesis, collagen genesis and arrival new bone. Bone cells, osteoblasts and osteoclasts can be seen on implants HAp / PLLA after six weeks of implantation. These results showed that HAp/PLLA tissue graft have good biological properties, like bone. Therefore, biocomposites HAp/PLLA can advertise by substitution unto bone in reparation defects of bone.

Introduction

Damage to the bone formed under different circumstances (e.g., fractures, trauma, infections, tumors). Balanced activity of bone cells, osteoblasts and osteoclasts is controlled absorption, regeneration, repair and bone growth. Therefore, the bone tissue can independently fix minor damage. However, with the emergence of major damage it is necessary to support the biological potential for bone tissue repair. For it the most commonly used are grafts and implants. In the United States only more than one million reimbursements of bone tissue are normally performed[1].

Autotransplantation is the gold standards in bone reparation of damage. However, the body is a little place where it is possible to take tissue for autotransplantation. Therefore, today are used allogeneic bone osteoinductive graphs, bone marrow cells and biomaterials as an

alternative autotransplantation. The possibility of using allograft and xenograft is limited in the case of histocompatibility. Today some natural or artificial materials are used as a substitute for the missing bone tissue[1-4].

Biomaterials

Biomaterials represent a wide group of natural or synthetic materials (synthetic polymers, biopolymers, metals, ceramics, and composites) which are used in medicine, dentistry, and veterinary medicine. Biomaterials are mainly used as components in medical devices (artificial joints, bones, teeth, heart valves, parts of blood vessels, artificial organs, artificial blood. For the application of biomaterials are of particular importance with tissue their biocompatibility, mechanical continuity with bone tissue, non-toxicity of biomaterials and their products after biodegradation of the organism and the lower price[2].

It is very difficult to classify biomaterials into the groups, subgroups and classes, because the same biomaterial can be defined differently based on different criteria of classification. On this ground biomaterials are divided into: metal (based on Ti, Co, Ni, Al, V...), polymer, ceramic, composite[3].

Polymeric biomaterials

Polymeric biomaterials represent a broad group of synthetic polymers which are characterized by structural stability, biocompatibility and biodegradation. Biodegradable polymers are capable of gradual degradation in the body, with non-toxic products that are easily excreted. They are used to create a system for the controlled release of drugs, carrier in tissue engineering, the fixation of bone fractures, surgical sutures[2] .The rate of degradation is determined by the chemical structure of the material, but most of all, by the kind and distribution of hydrophilic groups, reactivity of hydrolytic group distribution of amorphous and crystalline regions, porosity and molecular weight polymers. Until now mostly used were polyesters, polylactides and polyglycols[4]. The three biodegradable polymers possess good qualities in addition to composite materials they participate in building three-dimensional structure that provides an excellent basis for the growth of osteoblasts and deposition of mineral matrix. In recent times these composite materials are often added bioactive molecule type growth factors or certain cell lines[5.] Natural polymers such as collagen and hyaluronic acid play an important role in tissue engineering, mainly due to its distinct osteoconductive properties. In addition, they represent the natural tissue environment[4, 5].

Ceramics

Ceramics biomaterials are commonly used as part of the bone mineral imitators. Two types of ceramics are the most widely manual: β-tricalcium phosphate and synthetic hydroxyapatite (Ca_{10} $(PO_4)_6(OH)_2$). The main feature of these materials is their biocompatibility and osteoconductivity. Hydroxyapatite is absorbed more slowly than β-tricalcium phosphate; but hydroxyapatite they give more strength to the repaired tissue. Natural source of HAp for bone tissue regeneration are corals of the genus *Porites*. Ultrastructure of natural HAp has served to many researchers as a model for the creation of synthetic HAp[5].

Composite materials

Composite materials are solids which contain two or more distinct constituent materials. Natural biological materials tend to be composites. Natural composites include bone, wood, dentin, cartilage, and skin. Composite materials have adequate physical, biological and mechanical properties[2] .Many authors point to the importance of 3D structures of composite materials in osteoinduction[6, 7]. The improvement of biological characteristics of composite materials is possible by adding various bioactive molecules such as TGF, IGF, BMP, leptin, and parathyroid hormone[8].

Biofunctionality and biocompatibility are the two main characteristics that must be involved in the material to be used for medical purposes. Today, using for this purpose *in vitro* and *in vivo,* experimental approaches of a number of standardized experimental techniques are possible[2].
In vitro approach is based on the techniques of tissue culture, the isolation and monitoring of interactions between cells and biomaterials. These include: the culture of bone cells, culture of bone marrow cells, transformed and transformed cell lines and immortal osteoblasts cell lines[1-10].
In vivo experimental system includes the following models: filling bone defects, subcutaneous implantation of demineralized bone matrix or biomaterial[1-10].

Potential of HAp / PLLA composite in bone repair

Biocomposite HAp / PLLA produced a highly porous composite material as specified by the Institute of Technical Sciences of SASA. Composite biomaterial of HAp / PLLA with 80 wt% HAp and 20% PLLA-a 430 000 D has been used[9-11].

Bone marrow is complex tissue composed of hematopoietic and stromal mesenchyme stem cells (BMSC) with a differentiation potential to osteogenic, chondrogenic, fibroblastic, reticular, and adipogenic lineages. Therefore, the cultivation of bone marrow cells in culture is a good model for testing the proliferation and differentiation of cells under the influence of growth factors, hormones, and biomaterials.

In vitro testing

Bone marrow stromal cells have the ability to differentiate into mesenchymal cells that will later form a cartilaginous and the bone tissue. Many studies *in vitro* and *in vivo* have demonstrated a high osteogenic potential of BMSC cultured on calcium phosphate matrix[12-16].
One of the main factors in the osteogenic differentiation of BMSC cells is glucocorticoid dexamethasone[4,12-15] . Dexamethasone during differentiation induced morphological changes in cells. Cell shape changes so that the cells receive more or less cubical shape. In addition, this leads to increased expression of osteoblast markers such as alkaline phosphatase, osteopontin, and osteocalcin. Adding ascorbic acid enhances the osteogenic potential of BMSC *in vitro* and *in vivo* [12-14,17].

Tissue engineering is based on the development of biomaterials that are suitable for the cultivation of cells. If cultured cells, especially stem cells maintain their biological activity,

proliferation and differentiation on biomaterials, it can be expected that biomaterials can be used as matrices in tissue repair *in vivo*.

The analysis of BMS cell culture showed that cells adhere well to the Hap / PLLA[7,10,11,17]. Good adherence and cell migration on fibronectin substrate enables glycoprotein which is synthesized BMSC, and is also present in the serum for cultivation of cells[7,17]. The analysis of the distribution of cell types in culture BMS cells with biomaterial HAp / PLLA shows to reduce fibroblast cells. Reducing the density of cells cultured with HAp / PLLA can be explained by changes in pH. During the hydrolysis of PLLA leads to deliverance of H^+ ions, this reduces the pH of the environment. HAp component is unstable in acid and begins to dissolve by increasing the concentration of Ca^{2+} in the medium. Ca^{2+} ions stimulate cell adhesion to the biocomposite scaffold, and at the same time BMSC cell differentiation. These results indicate that HAP / PLLA suppress proliferation in favor of differentiation into osteoblasts[7,10,17].

In vivo testing

Studies of the interaction between cells and biomaterials, as well as organisms and biomaterials through different biological models are the basis for their use in tissue repair. Hydroxyapatite (HAp) is a good matrix for adherence, proliferation and differentiation. Ohgushi *et al*. have shown that HAp can serve as a matrix for culturing mesenchymal stem cells from the bone marrow, which would be under osteogenic conditions differentiate into osteoblasts. Such a matrix would *in vivo* possess the potential for the formation of new bone [18-20].

Bone formation *in vivo* is a complex process that may affect: (1) a biomaterial, (2) many environmental factors, such as. hormones, growth factors, etc., (3) and cells with osteogenic potential. Physical - chemical properties of biomaterials are of special interest for biomedical use. Type and form of the biomaterial, size, distribution and pore interconnectivity in biomaterials and biodegradation, are mostly responsible for osteogenesis[6,15,21,22]

Mechanical and degradation characteristics of the components of polylactide biocomposite type HAp / PLLA are caused by the combined effects of crystal structure and molecular weight of PLLA. The degradation of polylactide component involved two mechanisms: 1) easier diffusion of soluble oligomers from the surface of the medium, and 2) the neutralization of carboxylic groups on the surface. Consequence of these mechanisms is to reduce the acidity of the surface layer of biomaterials. Polylactide inside the biomaterial degrades faster because of auto catalytic process that starts with a terminal carboxyl group. Environmental conditions have a significant impact on the speed degradation of polylactides. In *in vivo* conditions process of degradation is carried out due to tissue enzymes, body fluids and cells surrounding the implant (Figure 1 and Figure 2). Given these facts, the degradation of HAp / PLLA is a complex multifactorial process[9-11].

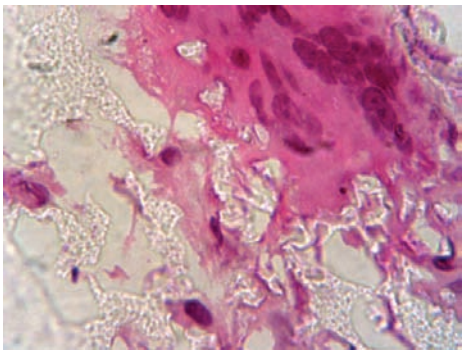

Figure 1. Degradation of HAp / PLLA with osteoclasts. HE 400x.

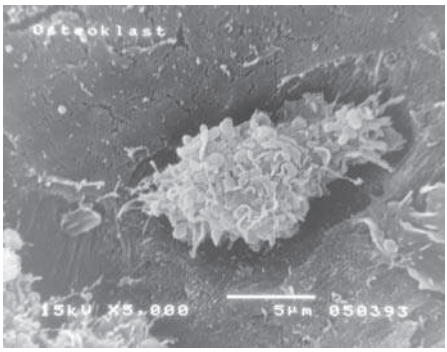

Figure 2. Degradation of HAp / PLLA with osteoclasts. SEM 5000x

HAp and PLLA induces an inflammatory response[21-23]. After one week of implantation around the implant HAP / PLLA fibrinous capsule is observed. Collagen fibers are spread through the biocomposite. This expansion is caused by the activity of mononuclear phagocytic cell, fibroblasts and reticular cells. First, mononuclear phagocytic cell phagocytosis HAP / PLLA and then reticular cells and fibroblasts synthesize collagen fibers. The results of our study show that fibroblasts exhibit their activity to form collagen fibers, so that after two weeks of implantation collagen fibers in the implant are formed. Activity of fibroblasts was significantly higher after six and twelve weeks after implantation, when the collagen fibers are present in large numbers and form bundles (Figure 3 and Figure 4).

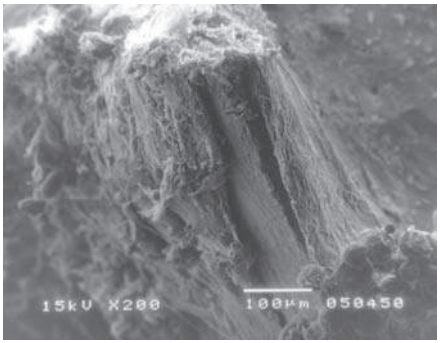

Figure 3. Fibrinous capsule around the implant HAP / PLLA. SEM 200x

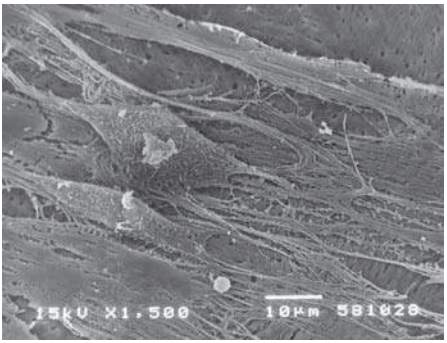

Figure 4. Activity of fibroblasts in collagen synthesis. SEM 1500x

Bone cells, osteoblasts and osteoclasts can be seen on implants HAp / PLLA after six weeks of implantation. Differentiation of bone cells is caused by existence of macrophage stimulating factor (M-CSF), ligand (RANKL), bone morphogenetic protein (BMP), which

is synthesized in mesenchymal stromal cells of the bone marrow[24-34] (Figure 5 and Figure 6). The presence of collagen fibrils with osteogenic cell elements is a prerequisite for the synthesis and mineralization of bone matrix this implies that the conditions for osteogenesis[27-35].

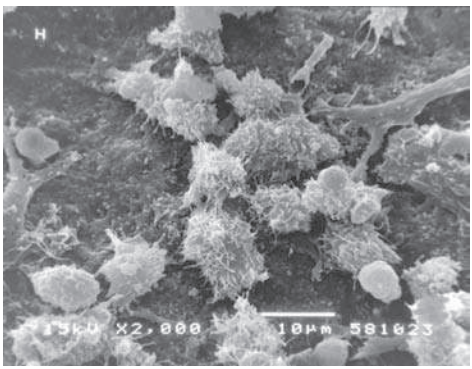

Figure 5. Different types of bone marrow cells in the composite HAp / PLLA. SEM 2000x

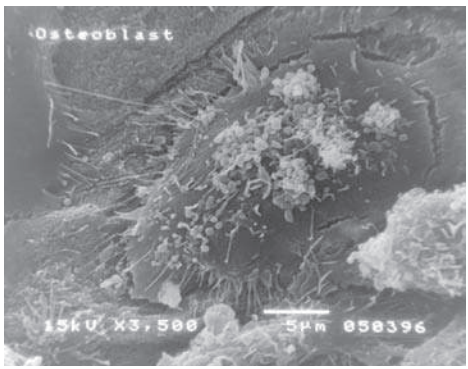

Figure 6. Osteogenesis in the composite HAp / PLLA . SEM 3500x

Conclusion

In vitro and *in vivo* analysis of biological characteristics of biocomposite materials HAp / PLLA biocomposite shows that HAp / PLLA has similar biological properties as well as the bone, it is recommended as a substitute for bone reparation of bone defects.

Acknowledgements

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Electrophoretic characterization of low-density and high-density lipoprotein subclasses and its significance in ischemic cardiovascular and cerebrovascular diseases

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Atherosclerosis-related diseases represent the main causes of morbidity and mortality worldwide. Identification of novel cardiovascular risk factors is therefore exceptionally important for primary prevention of such diseases. It is now widely accepted that traditional lipid parameters can not provide complete explanation for the role of dyslipidemia in the development of atherosclerosis. Analysis of low-density (LDL) and high-density (HDL) lipoprotein subclasses is recommended to ensure better assessment of cardiovascular risk. Several methods, including gradient gel electrophoresis, are available for LDL and HDL subclasses determination. In this paper we have analyzed electrophoretic characterization of LDL and HDL subclasses and clinical significance of their assessment in patients with ischemic cardiovascular and cerebrovascular diseases.

Introduction

Contemporary science firmly established the role of altered lipoprotein metabolism in the development of atherosclerosis-related diseases, such as coronary artery disease (CAD) and cerebrovascular disease (CVD). Disturbances of lipoprotein metabolism are primarily reflected as increased concentration of low-density lipoprotein (LDL) cholesterol with concomitant decrease in high-density (HDL) cholesterol concentration, which could be easily assessed by routine laboratory methods. Therefore, measurement of LDL-cholesterol (LDL-C) and HDL-cholesterol (HDL-C) concentrations in routine practice is recommended by international and national guidelines for prevention and treatment of atherosclerosis-related diseases[1]. However, there is an ample of evidence suggesting that an assessment of quality, rather than quantity, of LDL and HDL particles could provide more complete insight into patient's condition and, consequently, more accurate evaluation of risk for atherosclerosis development[2,3].

Qualitative analysis of lipoprotein particles comprises evaluation of their lipid and protein contents, which are reflected throughout changes in their size and density[4,5]. Populations of LDL and HDL particles are in fact heterogeneous mixtures assembled from different subclasses with variable sizes and densities, while precise subclasses distribution depends on general physiological and metabolic condition of each individual[4,5]. Numerous evidences have confirmed that distinct LDL and HDL subclasses could have more prominent role in initiation and progression of atherosclerosis. Accordingly, small, dense LDL and HDL subclasses have emerged as novel risk factors for CAD and CVD development[6-9]. However, the usefulness of their assessment in everyday clinical practice is still questionable, mostly due to methodological problems in lipoprotein subclasses analysis, but also due to uncertainty regarding their independent contribution to increase of cardiovascular risk[8,9].

Assessment of qualitative characteristics of LDL and HDL particles

During past few decades, several methods have been proposed for analysis of lipoprotein particles size and density (Table 1), all of which possessing significant advantages and weaknesses. Up to date, none of these analytical procedures have succeeded to gain broad and equivocal acceptance. Consequently, none of the available methods was declared as the reference one, so the question of standardization and comparison of results obtained by different procedures remains open.

Table 1. The most employed techniques for lipoprotein subclasses separation with corresponding resolved LDL and HDL subclasses

Employed technique	LDL subclasses	HDL subclasses
Density-gradient ultracentrifugation	LDL 1 LDL 2 LDL 3 LDL 4	HDL 2 HDL 3
Gradient gel electrophoresis	LDL I LDL IIa LDL IIb LDL IIIa LDL IIIb LDL IVa LDL IVb	HDL 2b HDL 2a HDL 3a HDL 3b HDL 3c
Nuclear magnetic resonance spectroscopy	L1 L2 L3 L4 L5	H1 H2 H3 H4 H5 H6 H7

Density-gradient ultracentrifugation was the first introduced method and till now, it has been widely employed. Another commonly used procedure is electrophoresis on nondenaturing polyacrylamide gradient gels (GGE), based on principle that lipoprotein particles migrate trough the gel until reach the exclusion limit determined by their size and shape. The gels are then stained for either lipids or proteins and scanned to obtain densitometric trace of the sample. Protein standards of known diameters are included on every gel for calibration. Several modifications and enhancements are introduced over the time, wherein one of the most significant improvements was the employment of composite gels for simultaneous separation of both LDL and HDL particles[10]. A method of 2D-electrophoresis was proposed for detailed analysis of HDL subclasses[11], while gel-tube electrophoresis was introduced as less expensive and more suitable alternative for separation and detection of LDL subclasses[12]. It is also worth mentioning that gels for analysis of LDL subclasses are now commercially available.

Finally, the most sophisticated method for qualitative analysis of lipoprotein subclasses is nuclear magnetic resonance spectroscopy[13]. However, high costs and employment of advanced technology strongly limit the use of this method in everyday practice.

Gradient gel electrophoresis for simultaneous separation of LDL and HDL subclasses

Initially employed electrophoretic techniques typically used two specialized 2-16% and 4-31% gradient gels for separate evaluation LDL and HDL subclasses. In an intention to facilitate the procedure, Rainwater et al. introduced a protocol for preparation of composite (3-31 %) polyacrylamide gradient gels, designed for simultaneous separation of LDL and HDL subclasses[10]. The same authors later applied several improvement of the original procedure[14]. This new methodological approach considerably enhanced the assessment of LDL and HDL subclasses, although a number of important drawbacks, such as extensive separation period, limited number of samples which can be processed at the same time, or extensive post-electrophoretic procedure still remained.

Simultaneous electrophoretic separation of LDL and HDL subclasses was introduced for the first time in Serbia several years ago at the Department of Medical Biochemistry of Faculty of Pharmacy, University of Belgrade[15,16]. We have modified the original procedure of Rainwater and co-workers[14] and adapted it according to our demands and laboratory conditions. Namely, we have increased the size of gels to achieve higher resolution of separated fractions, but also to facilitate handling the gels. For this purpose, we have developed a new gel casting protocol and, subsequently, investigated and established the optimal conditions for the separation of LDL and HDL subclasses. To facilitate the use of the method, we have simplified the visualization of separated fractions.

For preparation of composite (3-31%) polyacrylamide gradient gels in our laboratory we use Hoefer SE 615 gel caster (Amersham Pharmacia Biotech, Vienna, Austria), with 16 x 18 cm glass plates, 1.5 mm spacers and 15-well comb. Four gels can be cast at the

same time. Two computer-controlled peristaltic pumps (Masterflex L/S, Cole Parmer, IL, USA) are used for preparation of 12 gradient segments. The acrylamide solutions from the two pumps are collected and mixed in a home-made special mixer before entering into the casting chamber. Chemical polymerisation of the acrylamide monomers occurs at room temperature in the presence of ammonium persulphate and TEMED. Gels are calibrated by use of: carboxylated polystyrene microspheres (Duke Scientific, Palo Alto, CA, USA), Pharmacia High Molecular Weight protein standards containing thyroglobulin, ferritin, lactate dehydrogenase and albumin, alongside with human plasma with two LDL subclasses (diameters 26.99 and 25.52 nm), standardized in Dr. David Rainwater's laboratory (Southwest Foundation for Biomedical Research, San Antonio, TX, USA). Electrophoresis is performed at 8°C in Hoefer SE 600 Ruby electrophoresis unit (Amersham Pharmacia Biotech, Vienna, Austria) using Tris-boric acid-Na_2EDTA buffer, pH 8.35. Duration of this phase is approximately 20 hours. After completion of the electrophoresis, gels are stained overnight for lipids with Sudan black (Merck, Darmstadt, Germany) in ethanol and destained for 2h in ethanol. The lateral portions of the gels containing protein standards and latex beads are stained separately with Coomassie brilliant blue G-250 (Sigma, St. Louis, MO, USA) in ethanol-acetic acid-water solution. After destaining, gels are soaked in the buffer to resume their original rectangular shape. The gels are analysed using Image Scanner (Amersham Pharmacia Biotech, Vienna, Austria) with Image Quant software (version 5.2;1999; Molecular Dynamics), to obtain migration distances and relative distributions for each absorbance peak in LDL and HDL regions. For this purpose, previously defined regions with the cutoff values for every LDL and HDL subclass are used. In order to investigate correlation of our method with the initial electrophoretic method for assessing LDL size and subclasses developed by Krauss et al.[4], we have analyzed 24 plasma samples using both composite and special (2-16 %) LDL gel. Comparison analysis showed that our method was highly correlated with the reference one (Y-intercept was 1.14 ± 0.76, slope was 0.96 ± 0.03, $r^2 = 0.992$). The intra- and inter-assay coefficients of variations for LDL size were 0.3% and 1.1%, and for 2.2%, HDL size 1.8% and respectively ($N = 10$), as published previously[16].

During the time, several approaches were proposed for analysis of gels and evaluation of LDL and HDL subclasses. At the commencement, the most widely employed procedure was determination of the dominant particle diameter (nm), which denotes estimated diameter for the major (dominant) peak in LDL and HDL size region (Figure 1). According to the dominant diameter, it is possible to classify subjects as carriers of distinct LDL and HDL phenotypes. Namely, subjects with dominant LDL diameter ≥ 25.5 nm are classified as carriers of LDL A phenotype, while LDL B phenotype is assigned to individuals with dominant LDL diameter < 25.5 nm. Accordingly, HDL 2 phenotype is assigned to subjects whose dominant HDL diameter is higher or equal than 8.8 nm, whilst HDL 3 phenotype is referred to dominant HDL diameter < 8.8 nm.

However, the results of later researches suggested that more accurate evaluation of individual LDL and HDL profiles could be obtained by calculation of proportional subclass distribution

(%), which shows the fraction of the total area under curve accounted for each separate LDL and HDL subclass. By using these data, it is possible to achieve plasma LDL and HDL subclasses cholesterol concentration, which is derived by multiplying total LDL and HDL concentration by relative proportion of each subclass. Finally, by using predefined cutoff values of 25.5 and 8.8 nm, one can obtain relative proportions of small, dense LDL, or small-sized HDL subclasses, which is very simple and convenient mode for presenting lipoprotein profile, if we appreciate that these particular subclasses have the most important role in atherosclerosis development.

The most prominent advantage of simultaneous electrophoretic characterization of LDL and HDL subclasses is reflected by the fact that this method enables a broad and detailed insight into complete LDL and HDL profile. Having in mind that LDL and HDL share joined and cross-linked metabolic pathways and that remodeling of these particles occurs in a mutually dependent manner, it is clear that their simultaneous evaluation represents the optimal procedure. On the other hand, extensive and time-consuming process, a need for advanced equipment and experienced staff and toxicity of reagents are significant weaknesses. Nevertheless, the main disadvantage of this protocol is common for all methods for lipoprotein subclasses analysis: a lack of standardization and difficulties in comparison of results obtained by different procedures in different laboratories. Such drawback appreciably aggravates achieving definitive and reliable conclusion regarding the significance of lipoprotein subclasses in prediction and management of risk for development of atherosclerosis.

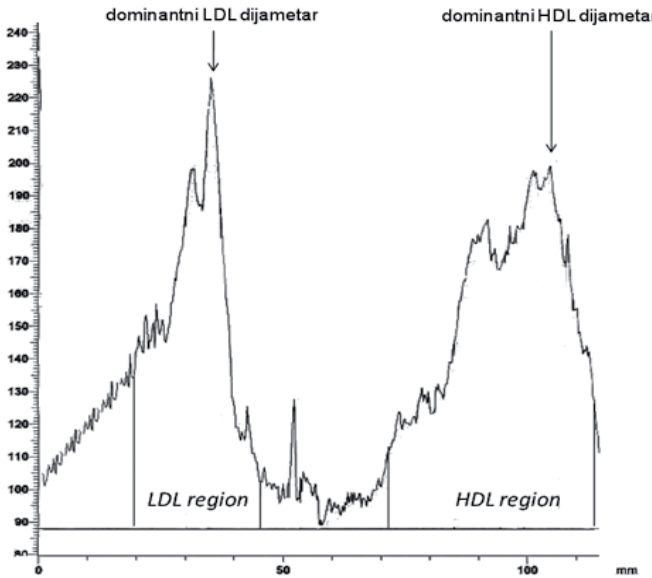

Figure 1. Densitometric scan of separated LDL and HDL subclasses

Significance of LDL and HDL subclasses determination in ischemic cardiovascular and cerebrovascular diseases

Atherosclerosis-related diseases are among leading causes of mortality and morbidity worldwide[17]. Therefore, identification and evaluation of markers which could be useful in prediction and management of the diseases is especially important. As it was previously mentioned, alterations in serum lipids play a pivotal role in development of atherosclerosis. Nowadays, it is widely accepted that the initiation and progression of atherosclerosis are caused by interaction of several crucial processes, including chronic low-grade inflammation, enhanced oxidative stress and disturbed lipoprotein metabolism[18-20]. It is well known that increased concentration of LDL-C is critical for formation and propagation of atherosclerotic plaque. However, newer evidences have suggested that the role of lipoproteins in atherosclerosis development mostly overcomes single effect of elevated LDL-C concentration[21]. More comprehensive understanding of this topic is provided through the term "atherogenic lipoprotein phenotype" or "atherogenic lipid triad", which comprises three lipid abnormalities, all regularly seen in patients with advanced atherosclerosis: increased concentration of triglycerides, decreased HDL-C concentration and increased presence of small, dense LDL particles[22]. Such approach, which includes not only quantity, but also quality of serum lipids, has widely opened the door to extensive investigation of lipoprotein subclasses.

Over the last few decades, LDL and HDL were the most comprehensively studied lipoproteins. As a result of numerous researches, small, dense LDL and small HDL particles have emerged as possible novel cardiovascular risk factors[8]. Atherogenic potential of small, dense LDL particles is based on their reduced clearance from the bloodstream, enhanced penetration through endothelial barrier, prolonged residence time in sub-endothelial space, propensity towards detrimental activities of free radicals and greater affinity for macrophage scavenger receptors[8]. Possible role of small HDL subclasses in atherogenesis is even more complex. Namely, small HDLs have marked capacity for reverse cholesterol transport and therefore, they are considered as potent atheroprotective particles[7]. However, it has been demonstrated that these particles are especially prone to harmful effects of hypertriglyceridemia, oxidative stress and inflammation, which are commonly seen in patients with atherosclerosis[5]. In such adverse environment, small HDLs can lose their atheroprotective abilities and even become pro-atherogenic[5]. Taken all together, changes in distribution and relative proportions of LDL and HDL subclasses are undoubtedly associated with the development of atherosclerosis.

Although the role of specific LDL and HDL subclasses in atherogenesis has been elucidated, a controversy related to their clinical significance still remains. The main question is whether small LDL and HDL subclasses are independent cardiovascular risk factors, or is the observed increase of cardiovascular risk merely a consequence of complex interactions with other traditional lipid risk factors? Numerous studies have demonstrated increased proportion of small, dense LDL particles in patients with CAD[23]. Up till now, more than 50 investigations of various designs have been conducted and waste majority of them have confirmed the

associations of small, dense LDL with development of the disease[23]. However, small LDL size also correlated with elevated concentrations of triglycerides, decreased HDL-C concentrations, as well as with few other non-lipid cardiovascular risk factors, such as adiposity and insulin resistance[24,25]. These complex metabolic inter-relationships challenge previously assumed independent contribution of small, dense LDL to CAD development. Indeed, results of multivariate analysis in most of the cases revealed that small, dense LDL particles were not independent predictors of CAD, suggesting that increase of risk was most likely a consequence of a broader pathology[23]. The fact that characterization of LDL subclasses was performed by different techniques in various studies further complicate reaching of final conclusion regarding the independent contribution of small, dense LDL to overall CAD risk[23].

In contrast to the ample of evidences about the role of small, dense LDL in CAD, there is lack of information regarding LDL subclasses distribution in CVD. Available data suggest that LDL size and subclasses distribution are associated with development of carotid lesions[26-29]. Independent contribution of small, dense LDL to the onset of CVD is still questionable, although Berneis et al.[30] have demonstrated that LDL size was strong and independent predictor of carotid artery intima media thickness.

Size and distribution of HDL subclasses and their effect on development of atherosclerosis have been extensively investigated, as well. Results of Framingham Offspring Study[31] and Veterans Affairs HDL Intervention Trial[32] have demonstrated significant shift of HDL particles distribution towards smaller subclasses in subjects with CAD. Similarly as in studies of LDL subclasses, there is still no relevant conclusion regarding independent involvement of small HDL particles in the disease progression. Likewise, the effects of altered HDL subclasses distribution on the onset of CVD are still insufficiently explored.

Results of our own research of LDL and HDL size and subclasses distribution in 181 patients with CAD[16] and 200 patients with acute ischemic stroke[33] have also demonstrated a shift towards smaller particles when compared to healthy controls. Furthermore, we have found that small, dense LDL particles were independent predictors of both CAD and ischemic stroke development[16,33]. In addition, small, dense LDL was identified as significant independent predictor of in-hospital mortality after stroke[33]. Therefore, our results confirm the assumption of independent effects of small LDL and HDL particles on initiation and development of atherosclerosis. In line with previous, we have also demonstrated altered LDL and HDL particles distribution in asymptomatic middle-aged subjects with elevated cardiovascular risk[15], but also in post-renal transplantation pediatric population[34], wherein we have found significant association of small HDL particles with elevated carotid intima media thickness, even without any other sign of early atherosclerosis. These later results further support the initiative to include LDL and HDL size and subclasses analysis to routine lipid measurements, at least for vulnerable populations.

In order to obtain a recommendation for routine assessment in everyday clinical practice, every new marker has to accomplish several prerequisites. An important requirement is

referred to clinical accuracy of novel marker that needs to be better than clinical accuracy of already processed parameters. Since this issue was scarcely investigated for LDL and HDL particles, we have performed such analysis. Our results suggested that clinical accuracy of simultaneous determination of LDL and HDL particles does not go beyond clinical accuracy of traditional lipid parameters in CAD[16]. However, in case of CVD, we have found a significant improvement of clinical accuracy after adding determination of small LDL and HDL subclasses to the measurement of traditional lipid parameters (Table 2).

Table 2. Receiver Operating Characteristic (ROC) Curves analysis of clinical accuracy of small LDL and HDL subclasses determination

	Area Under the Curve	Confidence interval	Standard error	P[a]
Model 1[§]	0,862*	0,820-0,898	0,021	
Model 2[§§]	0,730*	0,679-0,776	0,027	
Model 1 + increased presence of small LDL and HDL subclasses	0,898*	0,860-0,929	0,017	<0,01
Model 2 + increased presence of small LDL and HDL subclasses	0,809*	0,764-0,850	0,024	<0,001
Model 1 + Model 2	0,895*	0,857-0,926	0,018	
Model 1 + Model 2 + increased presence of small LDL and HDL subclasses	0,914*	0,878-0,942	0,016	<0,05

* Area Under the Curve significantly different from 0.5; § Model 1: age, smoking, hypertension; §§ Model 2: LDL-C, HDL-C, triglycerides; [a]P value for comparison of differences in AUC

Although the results presented herein strongly advocate the need for determination of LDL and HDL subclasses in high risk individuals, there are still many questions that have to be answered before the final verdict regarding clinical usefulness of this procedure could be made. One should have in mind that the presented results are mainly derived by using electrophoretic detection of lipoprotein subclasses, while there is still a problem with extrapolation of results obtained by different methodologies. Also, additional large prospective studies are needed to confirm or rule out the hypothesized independent role of particular LDL and HDL subclasses in the development of atherosclerosis.

Concluding remarks

In conclusion, simultaneous determination of LDL and HDL subclasses by GGE has several advantages over other available methods. The most prominent benefit is that this method provides comprehensive information about quality of particles, which are likely

to have significant role in the onset and propagation of atherosclerosis. However, the lack of standardization and difficulties in comparison of results obtained by different methods represent significant drawbacks. In addition, extensive use of this method is limited by its time-consuming and complicated protocol, as well as by shortage of adequately trained laboratory staff. Apart from technical limitations, clinical utility of lipoprotein subclasses determination is questionable, due to doubts regarding their independent contribution to overall increase of cardiovascular risk. Although numerous studies have confirmed intrinsic pro-atherogenic potential of small LDL and HDL subclasses, an agreement among investigators is not reached so far. Future perspective of LDL and HDL subclasses determination will depend on resolving both actual queries: technical issue and a question of its clinical significance.

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Electrochemical Analysis of Polyamino Acids and Proteins

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The aim of this review is the research of basic polyamino acids, such as polylysine, polyarginine, and polyhistidine as model systems of more complex proteins. The special emphasis is given to electrochemical contribution of individual amino acids in catalytic hydrogen evolution reaction (CHER), as well as investigation of CHER of proteins and carbohydrates.

In examining of polyamino acids, we concluded that they are satisfactorily good model systems to better understand the protein CHER. The electrochemical measurements were performed on mercury electrodes. It was shown that basic polylysine, polyarginine and polyhistidine produce chronopotentiometric peak H, while acidic polyglutamic acid gave no catalytic response.

In this presentation electrochemistry of DNA, proteins and osmium complex labeled polysaccharides and 3'-end ribose of RNA was shortly reviewed. Chronopotentiometric analyses enable precise determination of above-mentioned biomacromolecules at nano- and femtomole level.

For the first time histones were electrochemically measured. Investigation was performed at hanging mercury drop electrode (HMDE) and on glassy carbon electrode. It was shown that using constant current chronopotentiometric stripping analysis (CPSA) and cyclic voltammetry it is possible to detect histones at subnanomolar level. Also, for the first time electrochemistry has been used to investigate the interaction of histones with DNA. Above mentioned results show that electrochemistry is useful tool for precise determination of polyamino acids, proteins, nucleic acids, polysaccharides and histone proteins. This is a small contribution to a better understanding of role of individual amino acid in CHER at mercury electrodes. A very important part of so far unpublished results tell us that the CPSA can be used in research of interaction of basic histones with DNA in order to study of DNA packaging in chromatin. Findings from this review can also be used for testing of other nuclear non-histone and histone-like proteins, such as protamines and transcription factors.

Introduction – Bioelectrochemistry, Promising Tool for Research of Biomacromolecules

This review is based on a long-term research of peptides and proteins in our laboratory [1-11] and is predominantly focused on investigation of CHER of basic poly(aa)s, as model systems of histone proteins, on HMDE. Most of the proteins studied in our laboratory have acidic character, such as Riboflavin-binding protein (theoretical pI 5.1), Thioredoxin (theoretical pI 4.7), MutS protein (theoretical pI 5.5), α-Synuclein (theoretical pI 4.7), Serum albumin (theoretical pI 5.9), Concanavalin (theoretical pI 5.5), tumor suppressor p53 (theoretical pI 6.3), and many others. At the beginning of research represented in this work, we started to develop this research towards investigation of highly basic histone proteins (theoretical pI 10.3 – 11.4). To find suitable experimental conditions for histones study many factors were needed to be taken into consideration, such as purity, concentration and stability of dissolved tested proteins; purity, ionic strength and pH of supporting electrolyte; nature of protein adsorption on the electrode, the possibility of partial denaturation of proteins on the electrode, applied current/voltage, temperature, the presence/absence of oxygen in the system. Basic poly(aa)s as model systems for basic histones appeared to be suitable for better understanding of electrochemical behavior of histones. Thus our focus turned towards the studies of poly(aa)s as structurally simpler model of more complex histones. It was important to investigate how basic model systems of poly(aa)s adsorb at electrode and how they take part in the catalytic evolution of hydrogen. Using of poly(aa)s contributions of individual amino acid in CHER were studied. It is already well known that CHER is due to so-called *labile protons* of the tested analyte [12]. Basic amino acids, such as Lys, Arg and His, and sulfur containing Cys possess labile protons, so it was expected that polymers of these amino acids would catalyze CHER in a great extent. To our knowledge, we first showed the CHER of poly(aa)s on HMDE at weakly acidic and neutral pH [11]. Under our experimental conditions we concluded that acidic polyGlu gives no catalytic signal, as it was expected because of absence of labile protons, while polyArg,Trp yielded smaller catalytic signal compared to more basic polyLys. These findings give us an additional question: *How individual basic amino acid relatively contributes to the CHER on HMDE?* We investigated different homo poly(aa)s namely polyLys, polyArg and polyHis. Further we showed, for the first time, the different impact of Lys, Arg and His residues on catalysis [13]. Lys residues in polyLys give peak at the most negative potentials as compared to peak potentials of Arg residues in polyArg while His in polyHis yielded peak H at the least negative potentials. Also our preliminary results show that exchange of Arg to Lys in 14-mer bombesin peptides shifted of E_p to more negative values. Further study of influencing of E_p by aa residues is necessary. This type of study could be utilized in histone analysis, because individual histones H1-H4 have various aa composition (especially Lys and Arg content). Also we showed that besides CPS the cyclic voltammetry is suitable for study of poly(aa)s.

Diversity of histones – H2A, H2B, H3, H4 and linker histone H1 influence electrochemical response due to structure and amino acid composition. We showed that Cys-containing

histone H3 we differ from other histones because H3 yielded Brdi~ka catalytic reaction in agreements with previous results [14,15]. Also other non-Cys-containing histones can be discriminated on the peak potential, which depends mainly on aa residues. As it was mentioned above, experimental conditions strongly influence catalytical response. Further work is necessary for finding the better conditions for non-Cys-containing histones. But our work could be more difficult due to different structure of individual histones, what could strongly influence orientation of histone molecules at electrode surface and also catalytic process.

Protein–DNA interactions

DNA-protein binding plays a central role in many molecular processes in organisms and cells, such as replication, transcription, DNA repair and packaging. Research into the processes of DNA-protein binding and the nature and properties of complexes formed between DNA and proteins is therefore of utmost importance. Histones are proteins, which sequence specifically and also sequence nonspecifically interact with DNA. Non-specific interactions appear between positively charged helixes in H2B, H3, H4 with negatively charged phosphate groups [16,17]; histone chain amide groups form hydrogen bonds with DNA backbone [18]; histones form nonpolar interactions with deoxyribose of DNA [19]; lysine and arginine form salt bridges and hydrogen bonds with oxygens from DNA phosphate groups[19]; and where histones interact with DNA minor groove [20,21]. Sequence specific interactions were described in studies [22-24]. However, low DNA sequence specificity was estimated for the histone octamer [25].

Various methods including X-ray crystal analysis, NMR, fluorescence anisotropy, ultracentrifugation and other methods have been used to investigate DNA-protein interactions [26,27]. Only in recent years, methods of electrochemical analysis have been applied in DNA-protein interaction studies [28-30] dealing predominantly with aptamers (reviewed in [31-33]) mostly using labeled DNA. Recently label-free methods have been introduced and are based on changes in the electrode capacity (as detected by EIS) resulting from adsorption of a DNA-protein complex [34,35]. Preliminary results from our laboratory show that CPS analyses in combination with thiol-Hg-modified electrodes are suitable for study of protein-DNA interactions between p53 and DNA. Large differences in CPS responses of the sequence specific p53-DNA complex as compared to weaker non-specific p53CD binding to DNAs not containing the CON sequence were observed. To our knowledge, there are no records of using the electrochemical methods in investigation of histone-DNA interactions, and thus we conclude that electrochemistry might provide a contribution to this research.

Study of basic proteins

In addition to histones, there is still a whole range of other basic nuclear proteins, such as topoisomerases (theoretical pI 9.3), proteins of replication and transcription apparatus, gene repressors and activators, protamines (theoretical pI 12.1) and many other DNA interacting

nuclear proteins. The *histone-like* protamine proteins play an important role in DNA stabilization and packaging. Protamines are important, small, Arg-rich nuclear proteins that act as histones in sperm head condensation of DNA. It is believed that protamines bind to DNA more tightly than histones [36].

Nowadays it is known that every human cell contains many different proteins [37,38]. Many of these proteins have significant mutual structural analogy, which in turn depends on the function they perform. It is also known that proteins interact with their *structural domains* with other biomacromolecules and with low-molecular compounds. For example, protein-binding proteins possess domains *cadherin repeats* [39], *immunoglobulin domains* [40], *phosphotyrosine-binding domains* [41]; DNA-binding proteins possess *basic leucine zipper domain* [42], *zinc finger domains* [43]; Ca^{2+} binding *EF hand* domain [44]; ATP-binding proteins domains [45,46], and many others depending on the function. The examination of a large number of different proteins, finding conditions for their determination, research the behavior of proteins in solution, at interfaces and in various complexes may allow easier testing of, so far, electrochemically non-investigated proteins. In this sense, testing of homopoly(aa)s brought us some of the answers on easier determination of basic histones. Detailed examination of histones promises that it would be much easier to electrochemically investigate other basic histone-like proteins.

PolyLysine-Catalyzed Hydrogen Evolution at Mercury Electrodes

Poly(aa)s and peptides can be considered as model systems of proteins. Here, we studied electrochemical behavior of polyLys and two other poly(aa)s namely polyarginine,triptophane (polyArg,Trp) and polyglutamic acid (polyGlu) on mercury electrode [11]. At the first time we showed the role of individual amino acid residue in poly(aa)s in the CHER. At pH 6 polyLys and also polyArg,Trp yielded a peak H, in agreement with their ionization state, while polyGlu gave no catalytic response. Investigation of CHER of polyLys contributes to better understanding of electrochemical determination of peptides and proteins.

CHER of poly(aa)s was performed on hanging mercury drop electrode (HMDE) by methods of voltammetry and constant current chronopotentiometric stripping (CPS). 22.8 μM polyLys (related to the monomer content) at an accumulation potential E_A=–1.5 V and an accumulation time t_A=60 s in 0.1 M McIlvaine buffer, pH 6.0 produced well-developed CPS peak (peak H) at –1.88 V clearly distinguishable from the electrolyte discharge. Under the same conditions CPS of polyArg,Trp produced as about 20-fold smaller peak H than polyLys, while polyGlu yielded no catalytic signal (Figure 1). Chronopotentiograms confirmed that CHER needs so-called *labile protons* in amino acid side chain groups. Square wave voltammetric peaks of polyLys at –1.9 V were less separated from the background discharge compared to that obtained by CPS.

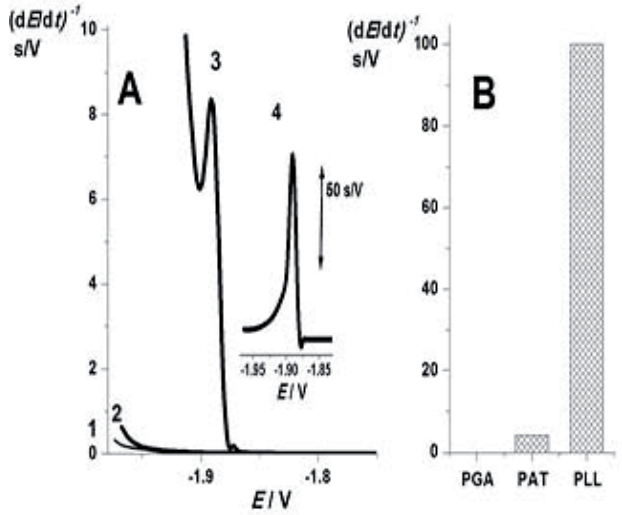

Figure 1 – A) AdS chronopotentiograms of 22.2 µM polyGlu (PGA) (2), 22.8 µM polyArg,Trp (PAT) (3) and 22.8 µM polyLys (PLL) (4, Inset) at HMDE recorded at I_{str}=–30 µA, t_A=60 s, E_A=–1.5 V, E_i= –0.1 V, E_f=–2 V in stirred solution. 1, background electrolyte 0.1 M McIlvaine buffer, pH 6. **B)** Bar graph showing relative intensities of peak H expressed as percent of polyLys signal intensity.

Investigation of catalysis shows that polyLys catalyzes HER in its adsorbed state. Moreover, by measuring of the effect of accumulation potential, E_A we can conclude that polyLys molecules could be absorbed by electrostatic or hydrophobic interactions with electrode surface.

In this work it is also noted that peak H is suitable and sensitive tool for studies of DNA-protein interactions. For this conclusion we tested the mixture of polyLys (5.7 µM) and DNA (5 µM) on HMDE (E_A=–0.5 V; t_A=120 s) in 0.1 M McIlvaine buffer pH 6.0. The peak of DNA/polyLys was of about 7-fold smaller than signal (peak H) of polyLys alone and by >100 mV more positive. These results show that peak H appears to be a new sensitive tool for studies of DNA-protein interactions. Probably similar approach might be used to investigate histone-DNA interactions.

Catalysis of Hydrogen Evolution by Polylysine, Polyarginine and Polyhistidine at Mercury Electrodes

Since the electrochemistry of peptides and proteins made significant progress in the past few decades, we believe that it is especially important to study the impact of each amino acid residue on the CHER. In this paper we studied contribution of polyLys, polyArg and

polyHis to CHER on mercury electrode [13]. In previous work with proteins, CPS was used predominantly, because voltammetric methods yielded poorly developed cathodic signals showing no relevance to changes in protein structures. Herein we showed that poly(aa)s produced well-developed voltammetric peaks.

Under experimental conditions shown on figure below, voltammograms are obtained in the presence of poly(aa)s and exhibit well-defined cathodic peaks at –1.89 V (polyLys, M_W of 4-15 kDa), –1.86 V (polyArg, M_W of 5-15 kDa) and at –1.70 V (polyHis, M_W of 5-15 kDa).

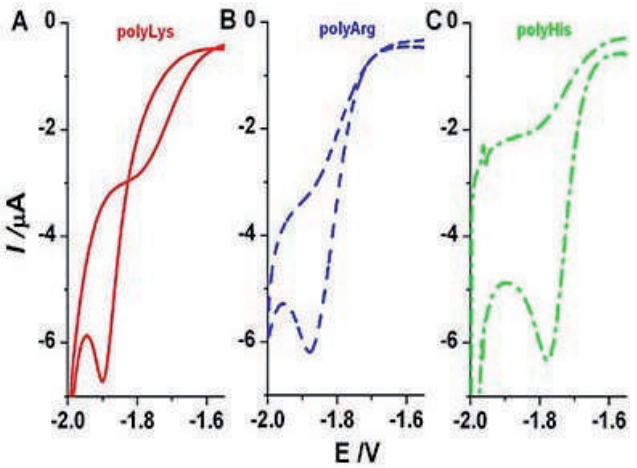

Figure 2 – **A-C.** Cyclic voltammograms of **A.** polyLys, **B.** polyArg and **C.** polyHis in 1/4 McIlvaine buffer, pH 7. 40 μM poly(aa) was adsorbed at HMDE at accumulation potential, E_A of –1.4 V for accumulation time, t_A of 120 s.

Investigation of I_{pc} dependence of polyLys of various lengths (M_W of 4-15 and 70-150 kDa) showed that shorter polyLys molecules move faster towards the electrode, so they earlier reach the electrode surface saturation. At electrode saturation voltammetric responses of those polyLys's were as of same extent and explanation is that the poly(aa) s chain lengths have no significant influence on the organization on electrode surface at full coverage. AC voltammetry has shown that these three poly(aa)s are adsorbed at the electrode in a different manner. Available literature suggests that under applied measuring conditions polyHis and polyLys adsorb as α-helix, while polyArg as random coil structured chain. Furthermore, in this study it is investigated the dependence of CHER on scan rate (ν). At low ν's, i.e. up to 50 mV/s, catalysis of poly(aa)s was as of the same extent. Greater difference is observed at higher scan rates, which suggests that relatively slow processes, such as diffusion of protons from the solution (to recombine with the aa residues in which reduction to hydrogen took place) could take place at slow ν, in agreement with the notion that electrode process coupled with chemical reaction

dominated at slow ν's. At greater ν's charge transfer more influenced the CHER. In CPS similarly to voltammetry at pH 7, polyLys peak H is the most negative (E_p –1.93 V) as compared to polyArg and polyHis. E_p of polyArg is –1.89 V, whereby polyHis peak is by 100 mV less negative as polyLys. The heights of poly(aa) peaks depended not only on the rate of potential changes but probably also by unequal length-distribution and structure of molecules in individual poly(aa)s.

This work shows that similarly as Arg in polyArg and Lys in polyLys also His residues in polyHis contribute to the catalysis of hydrogen evolution under the given conditions.

Electrochemical Sensing of Proteins and Carbohydrates

This work shortly reviewed about electrochemistry of DNA, proteins and peptides at mercury electrodes done in our laboratory [10]. Using of CPS enables us to investigate peptides and proteins at femtomole level. Also, in this paper it is presented the method of labeling of polysaccharides and 3'-end ribose of RNA by some osmium complexes and detected by CPS directly in reaction mixture at nano- and femtomole level.

First papers on electrochemistry of NAs were published about 50 years ago [47]. From that time until today electrochemistry of NAs was intensively developed including development of DNA biosensors capable of detecting even a single-base mismatch in DNA sequence. Recently, electrochemists turn their attention towards developing the methods for microRNAs estimation (shown to be potential tumor biomarkers).

Constant current chronopotentiometry in combination with mercury containing electrodes was successfully applied for study of peptides and proteins in distinguishing between native, denatured, aggregated, reduced/oxidized, mutant and chemically modified form of proteins by so-called peak H due to CHER. Besides the sensitivity to local and global protein structure, CPS analysis is suitable for detection of proteins at pico- to femtomolar concentrations.

Glycomics is started to be booming field. Alterations in protein glycosylation have been found for example in a variety of tumors. Using of electrochemical analysis in glycomics is a new approach in carbohydrates investigation. Carbohydrates contain neither chromophoric groups that strongly absorb UV/visible nor redox group for reversible electrochemistry. Therefore new approaches for carbohydrates detection are sought. We used complexes of six-valent osmium with nitrogenous ligands (L), Os(VI)L, for modification of some polysaccharides, and subsequently following electrochemical detection at mercury electrodes.

We hope that new electrochemical approaches described in this paper become useful in biomedicine, including neurodegenerative diseases and cancer research.

Catalysis of hydrogen evolution by histone H2A at mercury electrodes

Histones are small basic proteins containing a great number of basic amino acids (aa's), such as Arg and Lys. They are responsible for DNA stabilization and packaging into chromatin. To our knowledge, our work represents the first attempt of electrochemical investigation of histones. We focused on studying of histones H2A and H3. Also, we

performed some measurements with histone H1 and H4 (data not shown), but in much lesser extent as compared to H2A and H3. Our results show that histones H2A and H3 in neutral and slightly acidic pH produce catalytic hydrogen evolution reaction (CHER) at hanging mercury drop electrode (HMDE). CHER of histones, in particular, was performed by methods of CV and CPS analysis. Monitoring of irreversible oxidation of tyrosines and tryptophanes by means of square-wave voltammetry at glassy carbon electrode was also done.

First it was measured Brdi~ka catalytic responses (BCR) in cobalt-containing solutions showing that 1.5 µM histone H3 (containing one Cys residue) produce a well-measurable catalytic double wave, while 1.5 µM H2A (containing no Cys residues) only suppressed maximum of catalytic peak of cobalt (Figure 3A) without showing a double wave. Besides measuring of BCR, we turned our attention to investigation of chronopotentiometric peak H, for which Cys and also Arg, Lys and His residues are responsible for CHER at HMDE. Histone H2A and H3 are rich in basic aa's (~23-25% of total aa content) and 1.5 µM histones in 50 mM phosphate buffer, pH 7.3 produced a well-developed peak H for both H2A and H3 histones. Peak H of H2A was 2-times smaller than peak H of H3 and shifted to more negative potentials by about 60 mV (Figure 3C). Investigating these histones by CV we obtained similar results as with CPS analysis, i.e. peak potential of histone H2A was as about 70 mV more negative than peak potential of histone H3 (Figure 3B). Up to now CPS was predominantly used in protein study. Here we show for the first time that also CV is applicable for this purpose.

Our investigations showed that CV is suitable for distinguishing of histones by their different peak potentials (E_p), which greatly depend on Arg and Lys content. Our results show a relationship in E_p observed in CV measuring of polyArg and polyLys with peak potentials obtained for various histone proteins depending on their aa content.

CPS analysis proved to be a powerful tool for the examination of histone H2A in subpicomolar concentrations as well as for investigation of H2A-DNA interaction. Our results show a significant change in the size and potential of the peak H of H2A and H2A-DNA complexes at different ionic strength. Thus, these results suggest that the CPS analysis with HMDE could be suitable for investigation of protein-DNA interactions (especially for histone-DNA). Even though these interactions are intensively investigated by various non-electrochemical methods, electrochemical analysis has been only little utilized for this purpose. Further work will be necessary for better understanding protein-DNA interactions as detected at electrode surfaces.

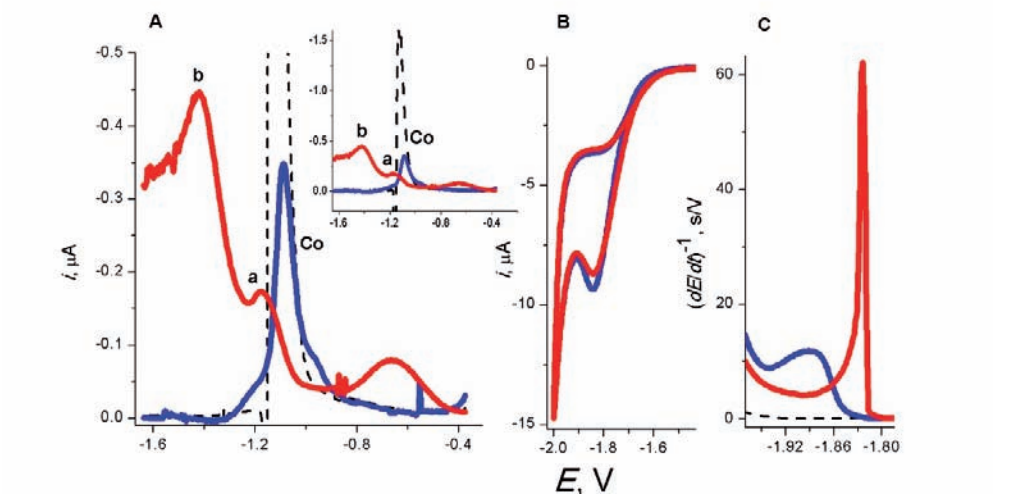

Figure 3. Stripping voltammograms (**A, B**) and chronopotentiogram (**C**) of 1.5 µM histone H2A (blue line) and histone H3 (red line) at HMDE in background electrolyte (dashed line) with adsorption time, t_A 60 s at a given accumulation potential. A) AdS differential pulse voltammetry (DPV): Background electrolyte of 0.1 M ammonium buffer with 1 mM $[Co(NH_3)_6]Cl_3$, pH 9.25 (BCR), frequency 223 Hz, step potential 5 mV, potential limit −1.85 V. a and b: catalytic protein peaks; Co: cobalt reduction peak. Inset A: The whole cobalt reduction peak range. B) AdTS cyclic voltammetry (CV): Background electrolyte 0.05 M sodium phosphate, pH 7.3, scan rate 0.1 V/s, initial potential −0.1 V, switching potential −2.0 V, step potential 2 mV, purging with argon 1 min before each measurement. C) *AdTS CPS*: Background electrolyte 0.05 M sodium phosphate, pH 7.3, stripping current −12 µA, potential limit −1.99 V.

Conclusion

Electrochemical analysis of proteins has recently entered a new phase based on contribution of CPS and bare and thiol-modified mercury electrodes which allow structure-sensitive analysis of various proteins, including those important in biomedicine.

In addition recent studies as well as some results contained in these presentation show that using CPS, interaction of proteins with DNA can be investigated. The basis of the above research lies on the ability of proteins to catalyze hydrogen evolution on mercury electrodes, discovered by J. Heyrovský et al in 1930. The outlooks of electrochemical analysis of proteins in 21th century appear great.

Summarized

This review focuses primarily on studying of poly(aa)s as a model systems of histone H2A. We tried to develop a new approach to the study of basic proteins and for the first time showed that histones and poly(aa)s are electroactive at HMDE, as well as that different amino acids make different contribution to CHER at HMDE. Studies were based on the methods of CPS and voltammetry.

- Poly(aa)s contribute to CHER at mercury electrodes by producing CPS peak H or voltammetric peak
- For the first time it was shown that individual amino acid residue in protein/peptide has its own role, regarding the influence on catalytic response directly by its own contribution to CHER or indirectly by influencing adsorption of the molecules
- Basic poly(aa)s produce peak H, while acidic poly(aa)s (such as polyGlu) give no electrochemical response under applied measuring conditions
- PolyLys catalyzes hydrogen evolution in its adsorbed state. PolyLys adsorbs at HMDE by electrostatic and also by hydrophobic interactions (even at potentials negative to the potential of zero charge)
- PolyLys can be considered as a model system of histones, which are rich in basic Lys and Arg residues
- PolyLys is useful tool for explanation of some principles important in analyzing of DNA interaction with basic histones
- Peak H potentials of individual poly(aa)s are different and depend on type of amino acid residues
- This work is the very first attempt for histone detection by means of CPS at HMDE
- CPS at HMDE can be used for investigation of histone-DNA interaction.

The results of this work indicate that it is possible and useful to use poly(aa)s as intermediate model systems between peptides and proteins for obtaining useful information about CHER on HMDE. It was especially important to use poly(aa)s for better understanding of the factors affecting CHER. Homopoly(aa)s proved to be beneficial for our attempts to clarify the role of individual amino acids in CHER, as well as for the simple approach to the study of the interaction of basic proteins with DNA using peak H.

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β-glucan-enriched extract ("Actiglucane") activates beneficial hepatic antioxidant and anti-inflammatory mechanisms in streptozotocin-induced diabetic rats

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Diabetes mellitus is a metabolic disorder resulting from defects in insulin secretion, insulin action, or both, that lead to chronic hyperglycaemia, the clinical hallmark of diabetes. Hyperglycaemia promotes the generation of reactive oxygen species (ROS) and disruption of antioxidant defence mechanisms, increasing oxidative stress which promotes the development and pathogenesis of various diabetic complications. Oxidative stress is causally associated with inflammatory processes in various diseases, including diabetes. The inflammatory process that accompanies diabetes is increasingly viewed as a pathogenic mechanism that promotes diabetic complications. Therefore, oxidative stress and inflammation, and the mechanisms they are comprised of, are important targets for therapeutic interventions in diabetes. Natural products are increasingly used to attenuate the complications of hyperglycaemia in diabetic patients. As the anti-diabetic effects of many compounds from plants such as polysaccharides have been documented, their effects are subjected to increasing scrutinization in different types of studies. Polymers of glucose, the β-glucans, are the most extensively studied class of plant-derived polysaccharides. In our research we investigated the protective potential of a β-glucan-enriched extract against the pathological processes in the liver of diabetic rats using the experimental model of multiple low-dose streptozotocin (STZ)-induced diabetes. We observed dual beneficial effects of the extract that were based on its anti-oxidative and anti-inflammatory properties. The obtained results shed light and support the usefulness of the β–glucan-enriched extract not only in the management of diabetes and its complications, but also in other pathological states of increased oxidative stress- and inflammation-related processes.

Introduction

Diabetes mellitus is a metabolic disorder characterized by hyperglycaemia resulting from disrupted insulin-signalling. The chronic elevation in glucose concentration and other metabolites elicits via different pathways, processes that cause a sharp increase in the

production of reactive oxygen species (ROS), depletion of the cell's antioxidant defence network and establishment of increased systemic oxidative stress[1]. ROS have been implicated in pancreatic β–cell destruction which underlies the pathogenesis of Type I (insulin-dependent) diabetes mellitus. Pancreatic oxidative stress is an important contributing factor in the onset and progression of diabetes[2], while the oxidative stress which accompanies the diabetic state plays an important role in the development of diabetic pathologies[1]. Therefore, diabetes is an ideal candidate to study the biological impact of oxidative stress as well as the potential beneficial consequences of its correction. The persisting redox imbalance in diabetes leads to the activation of stress-signalling pathways which trigger the expression of different inflammation-regulated genes[3,4]. It is becoming increasingly apparent that, in addition to promoting cytotoxicity, ROS may also initiate and/or increase inflammation. The oxidative stress-activated proinflammatory pathways represent a complex, interdependent and self-perpetuating pathogenic mechanism that, together with poor glycaemic control, promotes a variety of diabetic complications in different tissues and organs, including the liver[5].

The liver is the main organ of oxidative and detoxifying processes as well as free radical reactions, and is exposed to high levels of oxidative stress and subsequent ROS-mediated injury; liver oxidative stress can therefore be aggravated by diabetes-related metabolic disorders and itself negatively contribute to their development[6]. Hence, processes that contribute to oxidative stress are important targets for therapeutic interventions in diabetes management. In view of the rising incidence of diabetes and its long term complications, there is increasing demand for novel approaches in diabetes management that use natural products. The dietary intake of β–glucans, a diverse group of polysaccharides of D-glucose monomers linked by β–glycosidic bonds present in cell walls of fungi, yeast, oat, barley and bacteria[7,8], was found to provide considerable potential for diabetes management. By delaying stomach emptying, decreasing appetite and reducing food intake so that dietary glucose is absorbed more gradually[9], the β–glucans attenuate the postprandial rise in blood glucose levels[10,11]. While some reports have emphasized that the β–glucans stimulate immune function[12-14], proinflammatory activity[15-17] as well as the generation of ROS[18-20], there are studies in which the free radical scavenging activity of β–glucans[21-23], and their anti-inflammatory and anti-oxidative effects are documented[24-26].

In our research, we examined the effects of a commercially available β–glucan-enriched extract (obtained from barley, oat, asparagus, maize and *Pleurotus ostreatus,* with a dry substance ratio of 85:7:1:6:1; Essentia, Slovak Republic), on oxidative and inflammatory processes in the liver of rats subjected to the well-documented experimental model of multiple low-dose streptozotocin (STZ)-induced type 1 diabetes[27,28]. This model system has been used to study both the pathophysiological mechanisms of diabetes mellitus and the hypoglycemic activity of natural products[29]. The β–glucan-enriched extract was applied to the experimental animals through the daily intraperitoneal administration of 80 mg of the extract/kg for four weeks. Our results provide evidence that supports the documented anti-oxidative and anti-inflammatory effects of β–glucans.

In vitro free radical scavenging activities of the β–glucan-enriched extract

Earlier findings have questioned the scavenging activities of the β–glucans. Tsiapali et al. who examined the free radical scavenging activities of a variety of carbohydrate polymers concluded that the weak free radical scavenging activity of the examined glucan polymers could not explain the observed modulatory effect on the inflammatory responses in tissues, cell culture and disease models of inflammation[30]. Despite this result, increasing attention has been devoted to potential antioxidants of polysaccharide origin. The results presented in Kogan et al.[31] and our report[32] support the notion that the protective properties of the β–glucans could in part be ascribed to their free radical scavenging properties. β–glucans with free radical scavenging properties have been identified in some cereals and legumes and in mushrooms[33].

We evaluated different aspects of the antioxidant activity of the β–glucan-enriched extract. Increasing dose-dependent free radical scavenging activities were detected *in vitro* by the DPPH (1,1-diphenyl-2-picrylhydrazyl) radical-scavenging assay that has been widely used to evaluate the free radical scavenging effectiveness of antioxidant substances[32]. Also, the β–glucan-enriched extract exhibited a significant concentration-dependent metal chelating activity. The implication of this result is that the β–glucan-enriched extract acts as a metal chelating agent that functions as a secondary antioxidant which reduces the redox potential by stabilizing the oxidized form of metal ions. Furthermore, the β–glucan-enriched extract displayed a concentration-dependent capacity to scavenge hydrogen peroxide and the NO radical *in vitro*. Chronic production of NO is toxic to tissues and is associated with various pathological processes, including inflammatory conditions in juvenile diabetes. These results lend further support for an antioxidant activity of the β–glucan-enriched extract. They are in direct correlation with the findings that the antioxidant property of β–glucans protects macrophages from ionizing radiation, and restores bone marrow production[31]. The most often applied and investigated natural antioxidants are ascorbic acid, α-tocopherol and mannitol. According to literature data, the β–glucans are antioxidants whose scavenging ability is somewhere between that of α-tocopherol and the water-soluble antioxidant mannitol[34].

In vivo antioxidant activity of the β–glucan-enriched extract

Mechanisms that contribute to increased oxidative stress in diabetes include autoxidative glycosylation, nonenzymatic glycation, metabolic stress resulting from changes in energy metabolism, changes in the level of inflammatory mediators and impairments of endogenous antioxidant defence systems[35,36]. Disturbances of antioxidant defences in diabetes involve alterations in the activities of antioxidant enzymes. The activities of the major antioxidant enzymes, superoxide dismutase (SOD) and catalase (CAT), provide the first line of defence against increased ROS levels. Our results revealed that the activities of total SOD, CuZnSOD, MnSOD and CAT were decreased in the livers of diabetic animals, and that treatment with the β–glucan-enriched extract improved their activities[32].

The levels of antioxidant enzymes critically influence the susceptibility of various tissues to oxidative stress. They are associated with the onset of disease and also with the development of complications in diabetes[37,38]. ROS have been implicated in both pancreatic β–cell destruction which underlies the pathogenesis of Type I (insulin-dependent) diabetes mellitus, as well as in liver injury[39,40]. STZ damages pancreatic β–cells whose antioxidant competence is very low[41,42]. The low antioxidant capacity renders pancreatic β–cells vulnerable to STZ-induced oxidative stress that suppresses insulin gene transcription and glucose-stimulated insulin secretion, which represent pro-apoptotic events[43]. Consequently, the rate of insulin secretion and concentration of plasma insulin is diminished significantly, ultimately resulting in hyperglycaemia. In addition to pancreatic β–cells, supraphysiological glucose provokes oxidative stress in hepatocytes which can lead to hepatic tissue damages[44]. STZ-related hyperglycaemia which results from β–cell destruction augments liver damage through reactive free radicals that mediate lipid peroxidation of the hepatocellular membrane and lead to hepatic tissue injury[45]. Increased lipid peroxidation as consequence of oxidative stress, is usually measured indirectly by assessing the concentrations of its end-product, malondialdehyde (MDA), a compound frequently used to estimate the oxidant/antioxidant balance in diabetic patients[46]. Our results show that the hepatic MDA level increased significantly in diabetic rats as compared to the control group[32]. This is in accordance with literature data regarding the STZ-related elevation of hepatic MDA[47-49]. We observed that treatment with the β–glucan-enriched extract inhibited the rise in MDA in diabetic rats, pointing to its antioxidative potential *in vivo*.

Glutathione (GSH), the most prevalent low-molecular weight peptide antioxidant, plays a critical role in limiting the propagation of free-radical reactions which would otherwise result in extensive lipid peroxidation. GSH depletion in diabetics has been shown in experimental[35,50] and clinical studies[51]. According to literature data, the significant reduction in GSH levels promoted by STZ, leads to a reduction of the effectiveness of the antioxidant enzyme defence system, sensitizing the cells to ROS[52]. Glutathione is present in cells in both reduced (GSH) and oxidized (GSSG) forms. Because of the action of the NADPH-dependent enzyme GSSG reductase, under normal physiologic conditions, the cellular content of glutathione is predominantly in favour of GSH. However, as oxidative stress promotes a decrease in the GSH:GSSG ratio, this ratio has become a sensitive indicator of oxidative stress [53]. Our unpublished data support a decrease in the GSH:GSSG ratio in the livers of STZ-induced diabetic rats. It has been proposed that antioxidants which maintain the concentration of reduced GSH can prevent oxidative tissue damage by restoring cellular defence mechanisms and by blocking lipid peroxidation[54]. According to our unpublished results, treatment with the β–glucan-enriched extract significantly restored the GSH levels in diabetic rats. Thus, the antioxidant effects of the β–glucan-enriched extract we observed *in vitro* were also confirmed in *in vivo* experiments.

The cytoprotective effect of the β–glucan-enriched extract

Oxidative stress stimulates opposing pathways that signal cell survival and cell death[55]. A complex cross-talk between these pathways ultimately resolves cell fate. One of the most actively studied kinase pathways in basic research and drug development is the serine–threonine kinase Akt pathway. Akt is a downstream target of phosphatidylinositol-3 (PI-3) kinase, that plays a central role in signals that mediate cell growth and cell survival, and it is widely accepted that the activation of Akt kinase exerts a pro-survival effect in cells. Stimulation of Akt by phosphorylation inhibits cell death in part by phosphorylation of the Bcl-2-associated death promoter protein which then releases Bcl-2 to inhibit apoptosis, as well as by inhibiting the caspase activation cascade, i.e. activation of the apoptotic effector caspase-3[56]. Caspase-3 is responsible for the cleavage of DNA repair enzymes, DNA fragmentation factor, nuclear structural proteins and cytoskeletal proteins. Martin et al. showed that strong oxidants that generate intracellular ROS downregulate Akt kinase (by dephosphorylation), and activate caspase-3 by proteolysis of its inactive form, pro-caspase-3[55]. Several different types of antioxidants are capable of decreasing caspase-3-mediated apoptosis and reducing tissue injury[57]. Hsu et al. have shown that the β–glucan from *Ganoderma lucidum* exerts an anti-apoptotic effect on neutrophils through the Akt-regulated signaling pathway and inhibition of pro-caspase-3 proteolysis[56]. In support of these data is our observation that treatment of STZ-induced diabetic rats with the β–glucan-enriched extract increased Akt kinase activity and decreased pro-caspase-3 degradation. These results reveal an important cytoprotective effect of the administered β–glucan-enriched extract[32].

Aside from lipids and proteins, DNA is a very important target in oxidative stress-induced damage. In our experimental model we examined the levels of primary DNA damage in the liver by the alkaline comet assay. DNA damage was quantified by measuring the displacement between the genetic material contained inside the nuclear sphere (comet “head”) and the resulting comet “tail”. The “tail moment’ has been suggested to be an appropriate index of induced DNA damage (considering both the migration of the genetic material and the relative amount of DNA in the tail). The treatment with β–glucan-enriched extract significantly reduced the level of DNA damage that was detected in diabetic rats[32]. According to the result showing that the tail moment in the liver of STZ-induced diabetic rats treated with the β–glucan-enriched extract did not statistically differ from that in the non-diabetic control group, it could be concluded that the extract did not exert any apparent genotoxic effects in the liver. These results are in agreement with the recent finding that β–glucans do not exert either genotoxic or mutagenic effects while protecting against DNA damage by ROS capture[58]. A number of “functional foods”, including those that contain β–glucans, have recently been shown to protect human DNA against genotoxic effects, associated development of cancer and other chronic diseases[59].

The effect of the β–glucan-enriched extract on the inflammatory response in the liver of diabetic rats

Recent evidence strongly suggests that oxidative stress due to redox imbalance is causally associated with inflammatory processes in various diseases including diabetes[60]. Each form of injury or tissue disorder that precipitates in inflammatory response inevitably causes a protective response aimed at restoring homeostasis which is referred to as the acute-phase (AP) reaction, characterized by a multifold increase in synthesis of AP proteins[61,62]. Modifications in AP proteins plasma concentrations are largely dependent on their biosynthesis in the liver, and changes in their production are influenced by the effect on the hepatocytes by proinflammatory cytokines which are principal stimulators of AP proteins. The signal transducer and activator of transcription 3 (STAT3) protein was originally identified as the main transcription factor involved in the cytokine-dependent induction of AP protein synthesis in hepatocytes[63]. The AP proteins exert a number of protective functions in response to inflammatory stimuli, however, excessive or persistent overexpression of AP proteins during chronic illnesses such as diabetes mellitus can lead to tissue and organ damage[64]. A chronically augmented AP response may be a mechanism that explains many of the clinical and biochemical features of type 2 diabetes and its complications. There is an increasing body of evidence that correlates the hyperglycaemia and the diabetic phenotype with chronically elevated levels of AP proteins. Yokoyama et al.[65] reported that AP proteins are elevated in Type I diabetes, especially in albuminuric patients. The inflammatory markers, the AP protein C-reactive protein and interleukin-6 (IL-6), as well as fibrinogen and complement factor C3, AP proteins associated with coronary heart disease, are also increased in diabetes[66,67].

Our preliminary unpublished results suggest that administration of the β–glucan-enriched extract could diminish the AP response elicited by STZ-induced hyperglycaemia. Potential attenuation of the inflammatory AP response in the liver of diabetic rats points to the anti-inflammatory effect as a potential mechanism that mediates the beneficial action of the β–glucan-enriched extract. This preliminary result prompted us to speculate that the anti-inflammatory action of the β–glucan-enriched extract could be accomplished through modulation of key inflammatory mediators, such as the cytokines. The major physiological importance of anti-inflammatory cytokine IL-10 seems to be the limitation of inflammation[68]. Its relative deficiency is considered to be pathophysiologically relevant, while its overexpression might in fact contribute to the limitation of inflammation. IL-10 has been shown to prevent hepatocellular damage in a variety of models of liver injury, and it is believed that the hepatoprotective effect of IL-10 is mediated via its anti-inflammataory action [69,70]. It is assumed that treatment with IL-10 is beneficial in various inflammatory animal models including diabetes mellitus[68,71]. In support of this concept Kiho et al., Hoffman et al., and Masihi et al. have also reported that certain glucans exert anti-inflammatory responses[24-26].

Advanced Glycation End Products (AGE): the link between hyperglycemia, oxidative stress and inflammation in diabetes

The link between hyperglycemia, oxidative stress and inflammation during diabetes involves the accumulation of advanced glycation end products (AGE), formed by the nonenzymatic glycation and oxidation of proteins and lipids, that promote tissue injury[72-74]. While the glucose oxidation in diabetes is the primary source of free radicals, increased AGE generation under hyperglycaemic conditions (in the course of which toxic ROS intermediates are released) is a secondary source of pro-oxidant signals[75-77]. In our model system, the increased concentration of glucose in diabetes promoted non-enzymatic protein glycation, manifested as elevated concentrations of glycohaemoglobin (GlyHb) and glycated serum proteins[78]. The administration of the β–glucan-enriched extract to diabetic rats substantially lowered the hyperglycaemia and levels of glycated serum proteins and haemoglobin by its normoglycemic activity. The observed ability of the β–glucan-enriched extract to lower hyperglycemia is an important aspect of its beneficial biological effect. Our results mirror the findings obtained in animal experiments and clinical trials that describe the reduction of blood glucose levels by fungal and oat β–glucan preparations[79].

Examination of the *in vivo* outcome of β–glucan-enriched extract administration on protein glycation was accompanied by the findings that the β–glucan-enriched extract decreased the formation of fructosamines and total AGE in an *in vitro* glycation process[78]. We concluded that the β–glucan-enriched extract can bring about a decrease of AGE levels indirectly, through its ability to lower hyperglycaemia, and by a direct effect on the process of AGE formation.

An important property of certain reactive or precursor AGE is their ability to covalently crosslink proteins, thereby altering their structure and function, as observed in the cellular matrix, basement membranes and vessel-wall components. Other features of AGE are related to their interactions with a variety of cell-surface AGE-binding receptors that lead to cellular activation of pro-oxidant and pro-inflammatory events[80]. Thus, besides their well-known direct toxicity, AGE also exerts detrimental effect by interacting and upregulating their receptors, including the receptor for advanced glycation end products (RAGE). The interaction with RAGE causes intracellular changes, most notably the activation of the redox transcription factors nuclear factor-κB (NF-κB) which regulates the expression of a large number of genes involved in inflammation, including RAGE itself[74]. Therefore, the activation of RAGE perpetuates NF-κB activation and initiates a positive feedback loop resulting in sustained RAGE upregulation which has been proposed to set the stage for chronic cellular activation and tissue damage[81]. This complex process lies at the root of an ongoing pathogenic cell dysfunction that leads to diverse diabetic complications, such as retinopathy, neuropathy, nephropathy and osteoporosis[82-84]. Consequently, it has been argued that therapeutic interventions aimed at preventing the formation of AGE have a vital role in delaying the development of diabetic complications[85]**.** The study of *in vivo* uptake of ^{125}I-AGE rat serum albumin from blood demonstrated that the liver is a major site of

AGE protein sequestration in the rat[81]. *In vitro* and experimental studies have shown that the RAGE-ligand axis may be involved in proinflammatory and tissue-destructive processes in hepatic tissue injury. By blocking the activation of intracellular signals that induce the RAGE pathway, harmful inflammatory mechanisms can be limited, thereby facilitating repair of the injured liver[86]. Basta et al. have reviewed the experimental and clinical evidence supporting the hypothesis that ligand-RAGE interactions can alter liver function and that this axis is a potential molecular target for the control of liver injury[86].

To further unravel the mechanism of the specific response and underlying signalling events related to the hepatoprotective effect of β–glucan-enriched extract in diabetes, we focused on the RAGE/NF-κB pathway. Our preliminary results suggest that the β–glucan-enriched extract accomplished hepatoprotection via anti-oxidative and anti-inflammatory effects in the liver during diabetes, by blunting the RAGE/NF-κB pathway. This means that the RAGE/NF-κB axis could be at least one of the target pathways for the protective effect of the β–glucan-enriched extraxt against liver injury in diabetic rats. Namely, it is well known that some ligands will activate RAGE strongly while structurally similar ones do not seem to activate it at all[87,88]. These ligands could antagonize the activation of RAGE and prevent further transmission of proinflammatory stimuli. These data point to an important antagonism of this group of ligands or RAGE binding proteins which could counteract or modulate deleterious effects of AGE-RAGE interaction. Thus, one of the effects of the β–glucan-enriched extract could be exerted via a competing interaction of the β–glucan polysaharide with RAGE that prevents the binding of AGE. This interference could be responsible for the observed suppression of the inflammatory response and further oxidative stress generation, and subsequent tissue injury. However, the precise mechanism remains to be clarified and further studies are necessary in order to address and delineate the precise pathway of β–glucan-enriched extract action regarding the RAGE/NF-κB axis.

Conclusions

Intake of β–glucans has been shown to reduce diabetes risk factors and to delay the onset of diabetic complications. β–glucan-enriched extract administration to diabetic rats described herein improves hyperglycaemia and promotes a significant reduction in systemic levels of non-enzymatically glycated protein species (AGE precursors). *In vitro* and *in vivo* experiments suggest that free radical scavenging and antioxidant activities of the β–glucan-enriched extract, together with the improvement of antioxidant enzyme activities, play vital roles in alleviating the oxidative stress accompanying diabetes. Treatment with the β–glucan-enriched extract exerts a protective effect against DNA damage in the liver of diabetic rats. Activation of the Akt prosurvival pathway could play a vital role in mediating the beneficial cytoprotective effects of the β–glucan-enriched extract. The presented results shed light on the effectiveness of the β–glucan-enriched-extract in the management of diabetic complications, as well as of other pathological states with strong oxidative stress and inflammatory components.

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Signalling and mitogenic activity of insulin analogue X10

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In recent years human insulin analogues have been engineered with the aim to provide a more efficient, reproducible and convenient therapy for people with diabetes. For patients that have life-long treatment with insulin it is very important to benefit from novel insulin analogues. In addition, molecular modifications in human insulin should not compromise any safety issue. Special attention has been directed to the mitogenic properties of insulin analogues compared to those of human insulin. Mitogenic potential of certain insulin analogues may result from an enhanced affinity towards the insulin-like growth factor-I receptor (IGF-IR), the occupancy time on the insulin receptor (IR) or a combination of IGF-IR- and IR-mediated processes. Specific attention has been focused on a rapid-acting insulin analogue, X10, which was the first analogue used in clinical development but was withdrawn at an early stage following discovery of mammary tumours in female rats. A thorough understanding of X10's interactions with both the IGF-IR and the IR and its signalling pathways has provided a clearer picture regarding its metabolic and mitogenic effects.

Insulin and cancer

The possibility of an association between insulin and cancer has attracted intense research interest among cancer epidemiologists since cancers of the colon, breast and pancreas have all been associated with increased circulating levels of endogenous insulin in the non-diabetic population[1]. This might explain some of the overlap between cancer risk in diabetes, obesity and other conditions associated with insulin resistance. Type 1 diabetes mellitus shows an excess cancer risk of ~20%, but involves a different range of tumours. Type 2 diabetes is associated with three of the five leading causes of cancer mortality in the USA-carcinoma of the colon, pancreas and breast (excess risks for each cancer are ~30%, 50% and 20%, respectively)[2]. Growth of all these cancers is influenced by the insulin-IGF-I signalling axis, as a consequence of cancer risk and levels of circulating insulin.

Recent studies showed significant improvement in the therapy of type 1 and type 2 diabetes due to the introduction of different insulin analogues (rapid-acting and long-acting) into clinical use[3]. Advantages of rapid-acting insulin analogues compared with conventional insulin preparations are better blood glucose control, faster onset of action and the short duration time. All these effects have been described for both patients with type 1 and type 2 diabetes. However patients with type 2 diabetes are still associated with an increased risk of particular cancers. Different glucose-lowering treatments can modify the risk of developing cancer in different ways, bearing in mind that metformin can provide a protective effect whereas insulin can potentially stimulate tumour growth[4]. Three out of four epidemiological studies published in June 2009 suggested an association between insulin glargine (long-acting insulin analogue) and an increased incidence of cancer[5]. Therefore increased interest in the molecular safety of insulin analogues has been raised.

Exogenous insulin administration is the only therapy available for type 1 diabetes mellitus and forms part of the treatment of type 2 diabetes mellitus. Endogenous insulin secretion is composed of two distinct patterns of discharge: bolus (post-prandial) and basal (constitutive) secretions[6]. Both types are very important in the regulation of specific metabolic processes. Human insulin in the body exists as monomers, dimmers and hexamers which are self-associated in conjunction with zinc ions. This represents insulin storage within beta-cell vesicles that maintain basal insulin levels[7]. During a meal immediate dissociation into dimmers and monomers occurs and the monomers are biologically active forms that bind to the IR. This induces a rapid increase in plasma insulin, which reaches maximal level after 30 to 45 min and decreases to its basal level within 2 to 3h[8]. However, in diabetic patients injection of human insulin does not mimic this profile. In these patients insulin has a tendency to self-associate and it is found as hexamers when injected in the subcutaneous tissue. As a consequence of this, appearance of injected insulin in the blood is delayed and could cause post-prandial hyperglycaemia.

The development of insulin X10

In order to improve insulin therapy for diabetic patients, several insulin analogues have been developed to mimic the physiological plasma insulin profiles of endogenous insulin. Using molecular approaches two main groups of insulin analogues have been established: short-acting insulin analogues that dissociate more rapidly following injection and long-acting insulin analogues that show delayed absorption or prolonged duration of action[7]. Most of the structural modifications of insulin analogues are found in the C-terminus of the β-chain (B26-B30 region) of insulin, as this region does not participate in binding to the IR[9,10]. These modifications in insulin primary structure may cause different absorption rates in the circulation, different binding affinities and specificities for appropriate receptors (IR or IGF-IR), different duration of activation of receptor and at the end, increased/decreased metabolic or mitogenic effects compared to that of human insulin[11,12].

The first rapid-acting insulin analogue was developed by modification of position B28-29

and replacement of the histidine residue for a negatively charged aspartic acid at position B10 (insulin X10 or AspB10)[13]. The histidine residue at the B10 position was known to be involved in self-association of insulin molecules through the coordination of zinc ions by the imidazole side chains. This change from histidine to aspartic acid disrupts the ability of insulin molecules to self-associate into hexamers, even at concentrations of 100 U/ml. After subcutaneous injection into pigs, the time taken for 50% of the dose to be absorbed from the injection depot was found to be approximately half that of human insulin[14]. Apart from increasing rate of absorption, injection of insulin X10 causes a higher maximum concentration and a more potent biological action compared to insulin. After discovering that insulin X10 causes increased rate of absorption in pigs, the same phenomenon was seen in both healthy volunteers and patients with type 1 diabetes. Insulin X10 also demonstrated a more physiological plasma profile than soluble human insulin in a randomised crossover study in which 21 male patients with diabetes were monitored for 2 months[15]. X10 was responsible for significantly lower postprandial blood glucose levels in the morning, similar glucose levels at lunch and dinner, slightly higher glucose levels during the night, resulting in similar HbA1c levels achieved with insulin X10 and human insulin. At the end of the study not any side effects were reported. All the evidence was supporting the hypothesis that insulin X10 was promising new rapid-acting insulin analogue.

However, a 52 week study of insulin X10 supra-physiological treatment of rats found that insulin X10 dose-dependently increase the incidence of mammary gland adenocarcinomas in female Sprague-Dawley rats[16]. In the group exposed to the highest doses of insulin X10 (200 U kg^{-1} day^{-1}), 44% of rats developed benign tumours whereas 23% of rats developed malignant tumours after 1 year. Furthermore, pancreatic β-cell proliferation in transgenic mice was also observed when the mice were treated with insulin X10[17]. These early observations led to a series of experiments in which mitogenic potencies of insulin and insulin X10 were investigated and compared.

Molecular characteristics of insulin X10

Insulin and IGF-I are two peptides that share a high degree of homology in both primary and tertiary structure[18,19]. They signal through their transmembrane receptors, IR and IGF-IR, which belong to the tyrosine kinase superfamily. Insulin and IGF-I share a common overlapping binding site on the two receptors. IGF-IR is mainly involved in mediating most of biological actions of IGF-I and IGF-II but it also has important roles in tumour biology[20]. A commonly held view is that insulin primarily induces metabolic effects whereas IGF-I is thought to regulate more mitogenic processes[21]. Metabolic effects of insulin typically occur at low concentrations and manifest themselves soon after exposure to the hormone. In contrast, mitogenic effects of insulin occur at relatively high insulin concentrations and after long-term exposures. However, several studies showed that mitogenic actions of insulin can be mediated via the IR, but due to cross-talk between the IR and the IGF-IR, proliferative effects of insulin, at least in part, can be mediated by the IGF-IR[22,23]. Insulin X10 has a two-fold to three-fold increased binding affinity for the IR compared to human insulin

and is approximately ten-fold more potent than insulin in stimulating biological processes. However, when compared to its metabolic potency it seems that its mitogenic effects are more enhanced. Substitutions of amino acids in the B chain of insulin molecule (how insulin analogue X10 is made) resulted in increased structural homology with IGF-I and increased affinity for the IGF-IR[24]. Hansen and colleagues[25] revealed that increased mitogenic potency of X10 may not only be due to increased affinity for the IGF-IR, but may result from the fact that insulin X10 exhibits a slower "off-rate" from the IR than human insulin therefore eliciting prolonged occupancy of the IR and activation of the IR tyrosine kinase and phosphorylation of the intracellular located Shc protein. In addition to the increased receptor affinities, insulin X10 was shown to have an increased *in vitro* ability to stimulate cell growth and DNA synthesis. It remains to be determined how receptor binding characteristics of insulin X10 relate to its intracellular signalling and enhanced mitogenicity. A large body of knowledge exists about intracellular signalling by insulin, very little is known about how X10 signalling differs from canonical insulin signalling pathways. Because X10 is likely to be a cancer promoter rather than initiator[26,27], cancer cell lines are relevant for examining X10's hypermitogenic effects.

Insulin signalling pathways

Insulin signalling pathways are critical for the regulation of intracellular and blood glucose levels and the avoidance of diabetes. Circulating insulin interacts with its cognate receptor, which is a transmembrane tyrosine kinase, having the $\alpha 2\beta 2$ configuration. Insulin binding to the α subunits leads to a conformational change and stimulation of the insulin receptor kinase activity through autophosphorylation of the β subunit and the tyrosine phosphorylation of insulin receptor substrates (IRS) that serve as docking sites for downstream effector molecules[28]. IRS phosphorylates the SH2 domain of Shp2, a tyrosine phosphatase, and the SH3 domain of the adaptor molecule Grb2. Activated Grb2 recruits Sos1 that, in turn, activates the extracellular regulated protein kinase (ERK) signalling pathway. IRS also activates phosphoinositide 3-kinase (PI3K) through its SH2 domain, thus increasing the membrane concentration of phosphatidylinositol-3,4,5-trisphosphate (PIP_3). This, in turn, activates PI3K-dependent kinase-1 (PDK-1) and its downstream effector kinases PKB (protein Ser/Thr kinase B, also named Akt), mTOR, p70S6 kinase and the atypical isoforms of PKC (PKCζ/PKCλ). In summary, both signalling pathways mediate the metabolic and growth-promoting functions of insulin, such as translocation of vesicles containing GLUT4 glucose transporters from intracellular pools to the plasma membrane, stimulation of glycogen and protein synthesis and initiation of specific gene transcription.

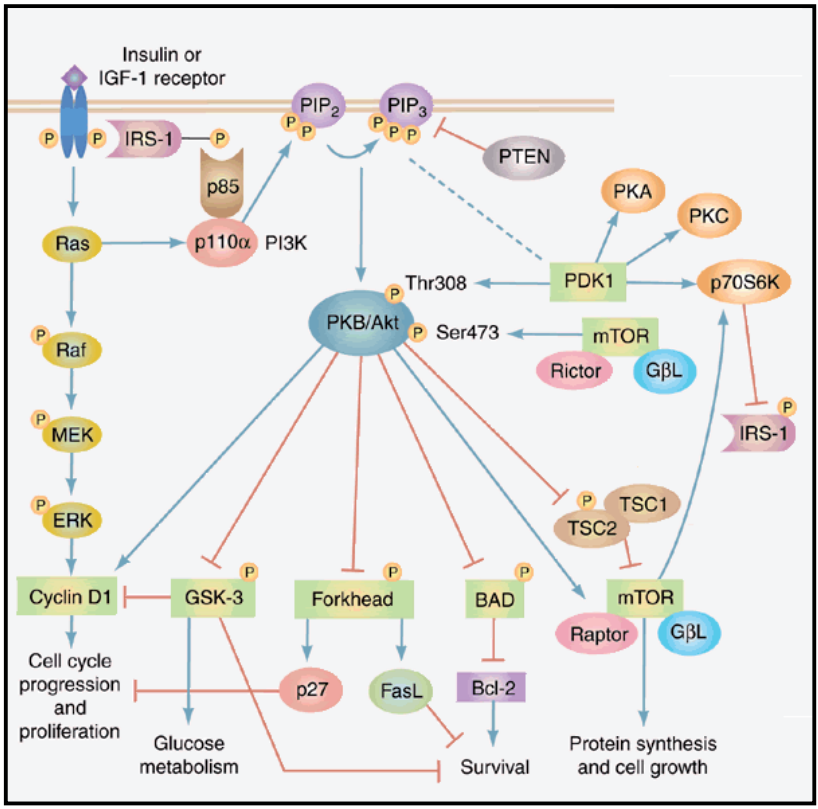

Figure 1. Insulin signalling pathways.

General overview on the mitogenic, proliferative and signalling effects of insulin X10

Up to date a number of reports examining possible connections between insulin X10 and its mitogenic activity in different cell lines have been published. In human breast cells (both MCF-10- a non-malignant human breast line and MCF-7- a human breast cancer cell line) insulin X10 has increased mitogenic potency and induces phenotypic changes as a consequence of its activation of both insulin and IGF-I receptors[29]. Oleksiewicz and colleagues revealed that in MCF-7 cells insulin X10 induces up to 2-fold higher phosphorylation of IRS-1, Akt, p70S6K, S6 ribosomal protein, 4E-BP1, FoxO3a, FoxO1, p44/42 MAPK and the EGFR compared to insulin[30]. The strongest difference in the intensity of phosphorylation after stimulation with insulin and insulin X10 was at the level of p70S6K. In order to obtain an adequate mitogenic response, the influence of insulin X10 was tested in human mammary

epithelial cells (HMEC) that express predominantly IGF-IR and in L6-myoblasts that were stably transfected to upregulate the level of IR[31]. In summary, the latter cells express ~200 times more IR compared to HMEC. Insulin X10 displays an increased relative mitogenic potency in both cell types indicating that it able to evoke an increased mitogenic response through both receptors.

Primary fibroblasts and smooth muscle cells from healthy donors were used to investigate the levels of IR and IGF-IR and downstream signalling due to the presence of human insulin, insulin X10, IGF-I, glargine, glulisine, lispro and aspart[32]. Using *in vitro* knockdown of IGF-IR and IR the authors provided direct evidence for a key role of the IR/ or IGF-IR/ Akt signalling pathway in the augmented growth-promoting activity of insulin analogues. IGF-I, X10 and glargine produced significantly stronger activation of Akt in primary fibroblasts compared to human insulin. This is in agreement with previously published data about the role of Akt in the regulation of DNA sytnthesis, cell proliferation and cell survival[33]. However, knockdown of IGF-IR by >95% resulted in substantially decreased Akt phosphorylation upon treatment with IGF-I, X10 and glargine. Probably the remaining signal is due to signalling through the IR. On the other hand, reducing the IR level by 75%, Akt phosphorylation stays at a high level only after acute stimulation with X10 and glargine whereas it is reduced by 40% in response to insulin. The remaining signal is probably due to incomplete knockdown of the IR. This was one of the first real pieces of evidence that the enhanced growth-promoting activity of glargine and X10 mediated by IGF-IR in primary human cells.

The newest *in vivo* data obtained from male Wistar rats treated with injections of human insulin, glargine and insulin X10 showed that activation of the IGF-IR could play an important role in its carcinogenic effects[34]. Insulin X10 stimulation resulted in at least 2-fold higher phosphorylation levels and significantly longer duration of IR and Akt phosphorylation in most tissues (muscle, liver and partially in abdominal adipose tissue). The authors concluded that in rats X10 has a different IR signalling profile to that of insulin and insulin glargine and that slightly elevated IGF-IR activity of X10 *in vitro* did not apply into IGF-IR phosphorylation *in vivo*. A possible hypothesis could be that greater mitogenic potency of X10 could be based on its altered IR profile *in vivo*. For the first time it became a possibly that IGF-IR activation by insulin analogues may be less relevant under therapeutic conditions than was previously thought.

Despite all the available data, X10's intracellular signalling, mitogenic activity and promotion of tumour growth are still not completely clear. If X10 binds to the IR or the IGF-IR, whether it activates PI3K or ERK signalling pathways or both of them, are still questions that have to be answered. Apart from the effects of insulin, X10 and IGF-I on proliferation of the colon cancer cell line HCT 116, very little data is available about X10's effects on other colon cancer cell lines. Weinstein and colleagues treated HCT 116 cells with regular insulin, IGF-I and the following insulin analogues: long-acting (glargine and detemir) and short-acting (lispro and aspart)[35]. Their results showed that IGF-I was the most potent stimulator of cell proliferation compared to stimulation with insulin. Treatment of HCT 116 cells for

12h with 100 nM IGF-I, glargine and detemir induced greater protection against apoptosis compared to insulin-treated cells and control. Insulin and glargine were responsible for the strongest phosphorylation of Akt whereas only insulin activated the ERK pathway. Yehezkel and colleagues performed very similar work in the same cell line as they treated the cells with human insulin, IGF-I, glargine and detemir[36]. They found dual activation of the IR and IGF-IR as a consequence of analogue treatment. Insulin glargine led to prolonged activation of the receptors that could induce abnormal signalling and was capable to induce IGF-IR internalisationto a similar extent to that of IGF-I.

Conclusion

We have achieved significant progress in understanding X10's binding to both IR and IGF-IR, its signalling through both receptors and the mechanism how it induces mitogenic effects. However, all these processes are not fully understood and investigations are still on going. Further clarifications of the remaining uncertainties and inconsistencies from previous research need to be assessed for safety reasons for new insulin analogue development. Future studies must employ more mammary cells in which intracellular signalling pathways will be investigating in depth hoping that the results will give answers why they are so important in malignant transformation and progression. As *in vitro* results go only so far in explaining *in vivo* effects, it will be advantageous to define a better tumour assessment model so that we can more accurately predict whether insulin analogues are likely to promote tumour growth. Despite the fact that on *in vivo* rat model that showed tumour-promoting effect of insulin X10 is still used today, it is not the best model for testing the long-acting insulin analogues because they will cause death as a consequence of hypoglycaemia. Currently other models that will bring more consistencies and more safety in this research area are under investigation (either models in which tumour formation is accelerated by induction with various chemicals or models in which tumours are transplanted into diabetic animals). Even the contribution of different IR isoforms and IR/IGF-IR hybrids in signalling processes of insulin analogues have been studied[37]. Available data show that both the binding affinity and the activation of the two IR isoforms are very similar after stimulation with insulin X10. However, the binding of insulin X10 to hybrid receptors is unknown at present and is under investigation.

Insulin analogue X10 was the first rapid-acting insulin analogue under clinical investigation and first results were very promising. Unfortunately, very quickly it was withdrawn from use. Despite this it is still a very important link in the current issue of the molecular safety of insulin analogues and is now recommended by the European Agency for the Evaluation of Medicinal Products as the positive control for insulin analogue mitogenicity studies.

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