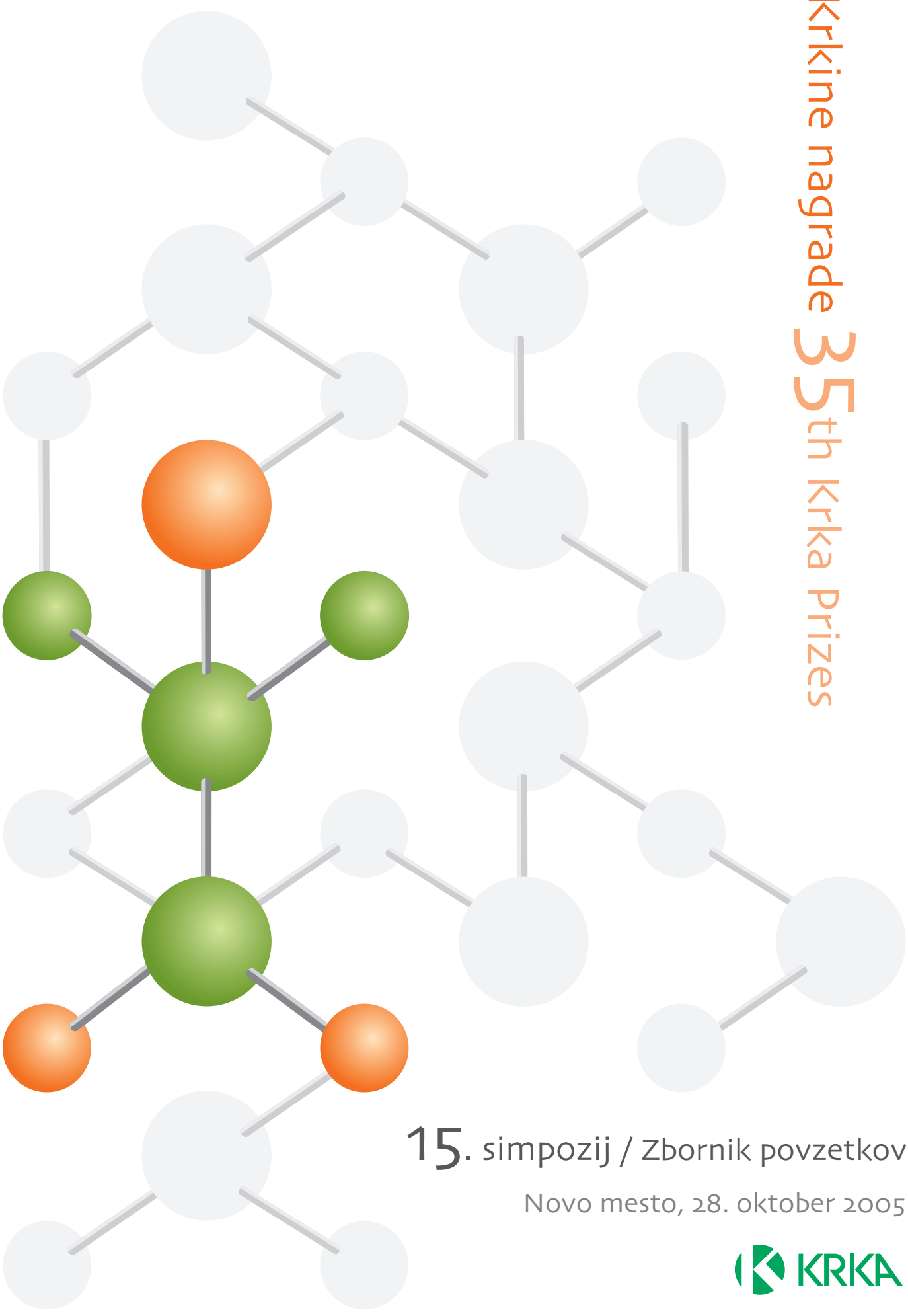
35. Krkine nagrade 35th Krka Prizes

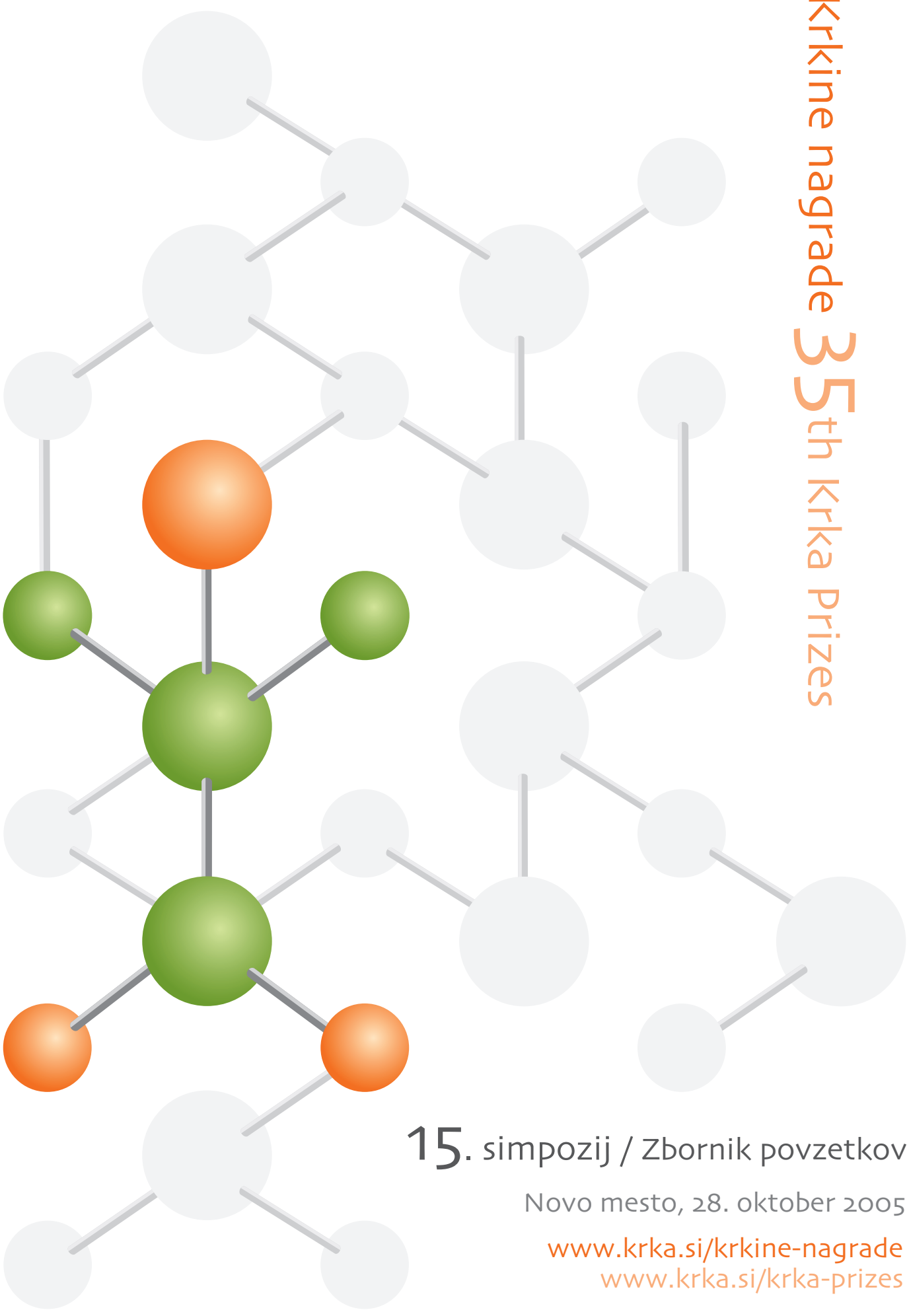


15. simpozij / Zbornik povzetkov

Novo mesto, 28. oktober 2005

KRKA

35. Krkine nagrade 35th Krka Prizes



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UVODNE BESEDE

Obdobje velikih sprememb v svetu, ki jim vsakodnevno sledimo, postavlja pred vse nas nove izzive. Na področju raziskav in razvoja je zadnja leta pronicljivemu opazovalcu postalo jasno, da se navidezne stalnice spreminjajo v spremenljivke. Če si zamislimo, kaj vse in kako raziskujemo ter razvijamo, pridemo do osupljivega spoznanja. Jedro našega razvoja je sposobnost posameznika, da na eni strani snuje in preizkuša nove ideje in išče rešitve na vprašanja. Enako pomembno je, seveda, da se pri tem povezuje s tistimi, ki mu na poti do cilja lahko pomagajo, predvsem pa, da razume, kje in kako se bo njegov rezultat uresničil. V zadnjem času pa je bolj kakor kdaj prej jasno, da se z dosego raziskovalnega cilja pot ne konča. Odgovornost raziskovalcev do javnosti je po dobi velikih odkritij in z njimi povezanimi še neuresničenimi pričakovanji danes večja kot kdajkoli prej. Čeprav je znanost naredila velike korake naprej, pa je največje spoznanje zadnjih let, da so posamezne panoge med seboj bistveno bolj povezane, bolj odvisne druga od druge in predvsem, da se medsebojno dopolnjujejo in ne tekmujejo. To spoznanje pa je še šibko in v senci neprestanega spraševanja o oblikah dela namesto o vsebinah.

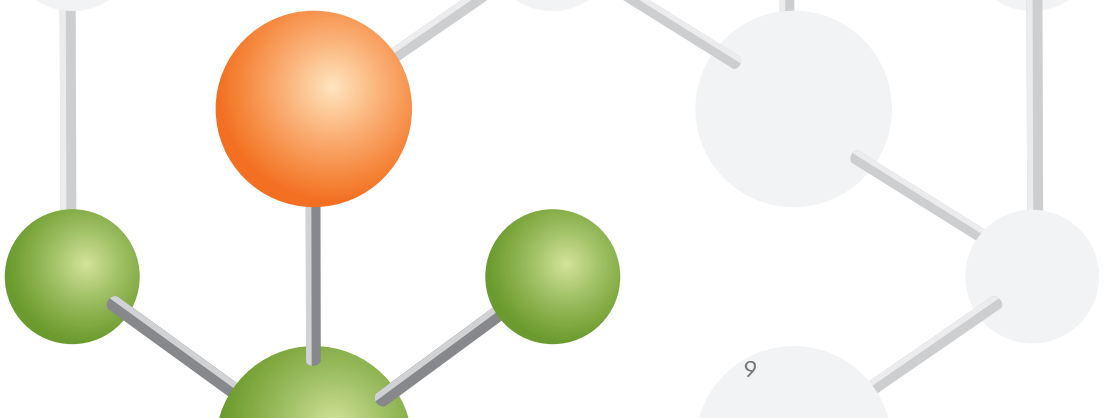
Pravo spoznanje soodvisnosti in potrebe po skladnem in v cilj usmerjenem delu prihaja s popolnoma drugega področja: iz vsakodnevnega življenja. Na našem področju, pri zdravilih, smo v zadnjem času doživeli tektonske premike. Vendar to niso bili veliki koraki naprej, ki so se obetali po epohalnih odkritjih, posebej na področjih blizu osnovnim mehanizmom življenja.

Veliki premiki so se zgodili pri naših pacientih, to je tudi pri nas samih. Ponovno smo pred nalogo, da utrdimo zaupanje v zdravila in zdravljenje. Tokrat nezaupanje ne prihaja zaradi zdravilnih pristopov drugih civilizacij, kot je to bilo v obdobju prodora alternativnih metod. Tudi ne prihaja iz zahodnjaških pristopov k duševnosti bolnika in iskanja ravnotežja med nezavednim in organizmom.

Prihaja zaradi nekaterih naših šibkosti, zaradi napak, ki so se dogajale pri raziskavah in hlastanju za novimi zdravili. Biti plat zvona ni znanstvenikom niti primerno niti pravilno. Predvsem moramo v sedanjem obdobju jasno ugotoviti, kaj je res dosegljivo, tako znanstveno kot gmotno, za najboljše zdravje posameznikov in družbe. To lahko dosežemo tako z vračanjem h koreninam farmacevtske, kemijske, medicinskih in sorodnih panog znanosti. Vsako res pomembno spoznanje bo pri tem imelo svoj prispevek. Farmacija lahko do rezultatov pride le z roko v roki z medicino. Biotehnologija je partnerica kemiji pri utrjevanje znanja o delovanju zdravil, ne pa njen konkurent.

Ključne besede v tem obdobju ne bomo našli v naboru le-teh v znanstveni literaturi. Zaupanje navadno ni tema znanstvenih člankov. Je njihova podlaga. Za uspešno delo, je potrebno zaupati, najprej sebi, svojim kolegom, svojim mentorjem. Še pomembnejše, žal marsikje novo, je zaupanje v druge raziskovalne skupine, saj je to predpogoj za sodelovanje. Zlasti pomembno pa je zaupanje drugih v nas. Če seveda lahko rečemo pacientom in zdravnikom drugi. Pravzaprav so samo oni prvi.

Graditi zaupanje z odličnimi dosežki je zato najpomembnejši cilj vseh, ki na področju raziskav delamo.



Dr. Aleš Rotar
predsednik Sveta
Sklada Krkinih nagrad

OPENING WORDS

An era of great change that we are faced with brings a new challenge to all of us every day. In the recent years any subtle observer has become aware that apparent constants in the field of research and development have turned into variables. If we just think of what can be investigated and developed and how, we come to an amazing conclusion. The very core of our development is an individual's ability to plan and test new ideas on the one side, and search for adequate solutions on the other side. It is equally important that he/she cooperates with the persons who can help him/her reach the goal, and particularly that he/she is well aware of how and where the relevant results will be implemented. It is more obvious nowadays than in the past that reaching a certain research goal does not mean that our task is completed. Investigators' responsibility to the public is nowadays, following the era of great discoveries and relevant unrealised expectations, greater than ever. Although science made huge steps forward, we have in the recent years become more and more aware of the fact that individual branches are inter-related much more than we used to believe, they are also more inter-dependent, and above all they do not compete, but mutually complement each other. This notion is still weak and overshadowed by constant queries about the form instead of about the substance of work.

The first idea about inter-dependence and the need for harmonious, goal-oriented action comes from an entirely different sphere: from everyday life. In our field of medicinal products, we have lately witnessed some tectonic shifts. However, they did not bring big steps forward, although they were expected to after certain epoch-making discoveries, particularly in the fields close to basic life mechanisms.

Great moves were observed in our patients, which means in us as well. We are again facing the task of consolidating the confidence in medicaments and therapies. This time the reason for lack of confidence is not in medical approaches practised in other civilisations, as was the case with emerging alternative methods. The reason can also not be found in western approaches to the patient's mentality and in trying to find a balance between the unconscious and the physical.

The reason lies in certain weaknesses and mistakes made in our studies and in our haste for new medicaments. It is not appropriate or correct for scientists to ring the alarm bell. It is our job nowadays to first realise what is actually attainable, from scientific as well as material perspective, for the best possible health of individuals and society. This can be done by looking back to the roots of pharmaceutical, chemical, medical and related branches of science. Any important newly acquired knowledge will contribute its share. Pharmacy can reach the results only by working hand in hand with medicine. Biotechnology and chemistry are partners and not competitors in consolidating their knowledge with regard to the action of medicinal products.

We shall nowadays not find key words in scientific literature, since confidence is usually not a topic of scientific articles. It is however their basis. If we want to be successful, we must first confide in ourselves, our colleagues and our mentors. Even more important is our confidence in other researcher teams, which is a precondition for cooperation, but is still rare to find and new. And it is very important that others confide in us. If the word others can be used for our patients and physicians. Actually, they are the first in the line.

To build confidence by presenting excellent achievements is therefore the utmost goal of everybody involved in the field of research.

Dr. Aleš Rotar
predsednik Sveta
Sklada Krkinih nagrad

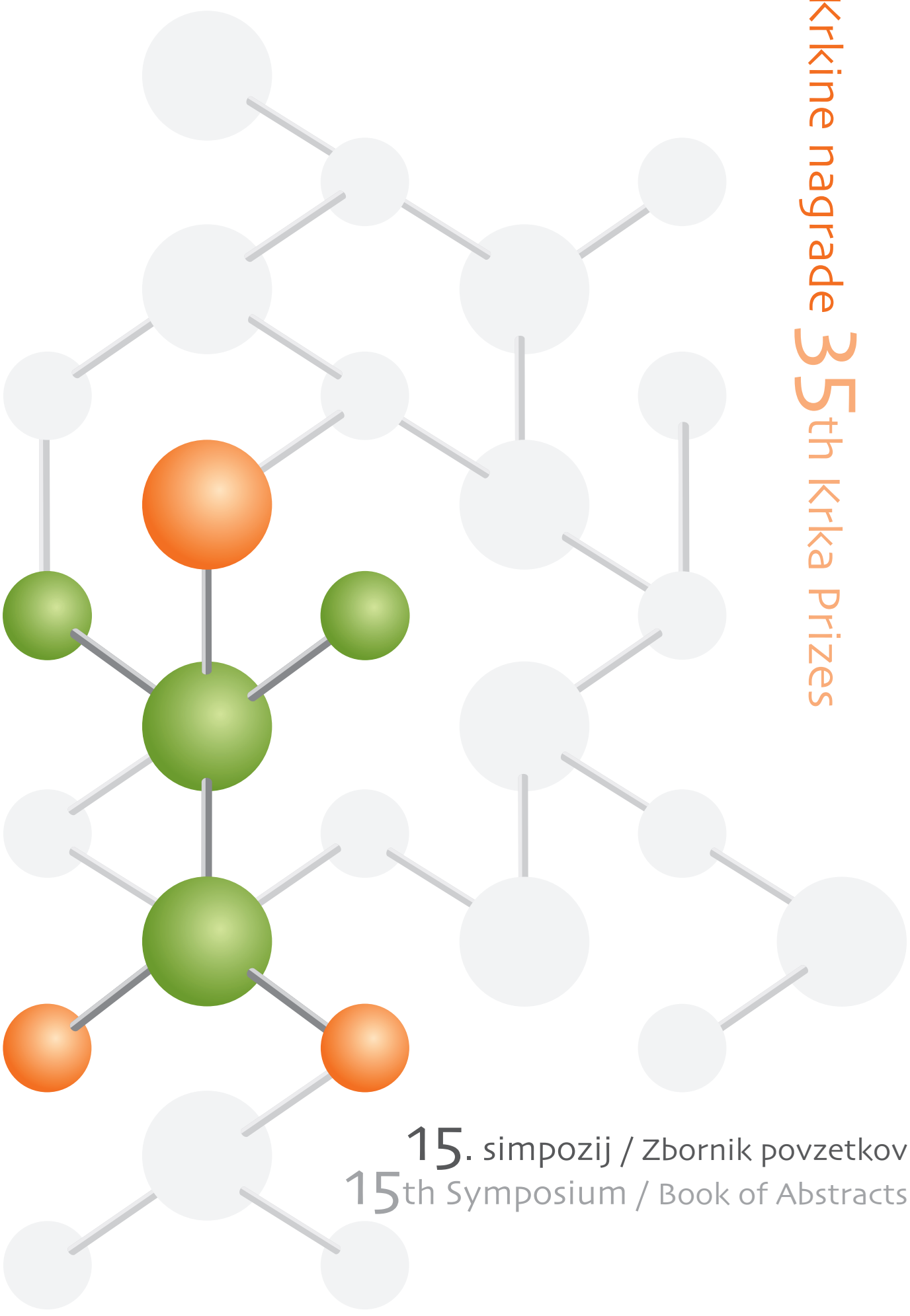
KRKINI NAGRAJENCI 2005 / KRKA PRIZE WINNERS 2005

Št.	Nagrajenci	Status	Mentor / Somentor(ji)
2053.	Marko Anderluh	doktorand	Marija Sollner Dolenc
2054.	Sergeja Bombek	doktorandka	Slovenko Polanc
2055.	Mojca Brunskole	študentka medicine	Katja Kristan Stanislav Gobec
2056.	Neža Čadež	doktorandka	Peter Raspor
2057.	Tadej Čepeljnik	doktorand	Romana Marinšek Logar
2058.	Rok Dreu	doktorand	Stane Srčič Iztok Žun
2059.	Aleša Dular	dijakinja	Marinka Kovač
2060.	Klementina Fon Tacer	doktorandka	Damjana Rozman
2061.	Barbara Golob	študentka farmacije	Stanislav Gobec Tea Lanišnik Rižner
2062.	Nataša Gomboc	študentka farmacije	Stanislav Gobec Tea Lanišnik Rižner
2063.	Petra Hudler	doktorandka	Radovan Komel
2064.	Maja Ilec	študentka Fakultete za družbene vede	Samo Kropivnik Tanja Kamin
2065.	Andreja Jarc	podiplomska študentka elektrotehnike	Stanislav Kovačič Markus Eblenkamp
2066.	Alja Jedlovčnik	študentka farmacije	Janja Marc Andrej Zavratnik
2067.	Saša Jenko Kokalj	doktorandka	Dušan Turk Gregor Gunčar
2068.	Mateja Jeras	študentka Fakultete za družbene vede	Samo Kropivnik Tanja Kamin
2069.	Martina Jerman	študentka kemije	Jana Zagorc-Končan
2070.	Mateja Jugovic	študentka Fakultete za družbene vede	Samo Kropivnik Tanja Kamin
2071.	Simona Jurković	študentka farmacije	Slavko Pečar Marija Sollner Dolenc
2072.	Urška Juršič	študentka kemije	Lucija Zupančič-Kralj Anita Mlakar

2073.	Matej Kastelic	študent medicine	Maša Goršič Radovan Komel
2074.	Marina Klarič	študentka Fakultete za družbene vede	Samo Kropivnik Tanja Kamin
2075.	Janja Kocjan	dijakinja	Jana Goršin Fabjan
2076.	Klemen Korasa	dijak	Aleš Gasparič Stanka Florjančič
2077.	Nina Kordež	študentka Fakultete za družbene vede	Samo Kropivnik Tanja Kamin
2078.	Urša Kovač	dijakinja	Stanka Florjančič
2079.	Katja Kristan	doktorandka	Tea Lanišnik Rižner Jure Stojan
2080.	Andrej Majcen	dijak	Stanka Florjančič
2081.	Petra Malavašič	dijakinja	Jana Goršin Fabjan
2082.	Mateja Manček Keber	doktorandka	Roman Jerala
2083.	Martina Marič	študentka Biotehniške fakultete	Nina Gunde-Cimerman Roman Jerala
2084.	Mitja Martelanc	študent kemije	Marijan Kočevar
2085.	Igor Milek	doktorand	Nataša Poklar Ulrih Peter Raspor
2086.	Marko Oblak	doktorand	Tomaž Šolmajer
2087.	Nina Perossa	študentka Fakultete za družbene vede	Samo Kropivnik Tanja Kamin
2088.	Miha Plevnik	doktorand	Janez Plavec
2089.	Igor Pokorny	dijak	Aleš Gasparič Stanka Florjančič
2090.	Mateja Primožič	doktorandka	Maja Habulin Željko Knez
2091.	Matej Reberšek	študent elektrotehnike	Damijan Miklavčič
2092.	Štefan Starčević	študent medicine	Katja Kristan Stanislav Gobec
2093.	Barbara Starič	dijakinja	Stanka Florjančič
2094.	Rok Šibanc	študent farmacije	Mirjana Gašperlin Filip Podlogar

2095.	Jerica Škedelj	dijakinja	Marinka Kovač
2096.	Marko Štefanič	študent farmacije	Albin Kristl
2097.	Matija Tomšič	doktorand	Andrej Jamnik Marija Bešter-Rogač
2098.	Ivan Toplak	doktorand	Jože Grom Darja Barlič-Maganja
2099.	Nina Zbašnik	dijakinja	Jana Goršin Fabjan

35. Krkine nagrade 35th Krka Prizes



15. simpozij / Zbornik povzetkov
15th Symposium / Book of Abstracts

Z MAJHNIMI KORAKI DO ZVEZD

Znanje je rezultat dela, tudi lastne urejenosti, ni odveč zavzetost, nadarjenost in vspodbujajoče intelektualno okolje. V Krki spremljamo in podpiramo predvsem uporabno znanje za tekmovalno soočanje s svetom. Letošnje nagrade in nagrajenci so izbrani najboljši med dobrimi, celo odličnimi.

Za znanstveni odbor
Prof. dr. Jože Drinovec

WITH SMALL STEPS TO THE STARS

Knowledge comes from working, inner orderliness, commitment and talent, stimulated by an intellectual milieu. In Krka we follow and support primarily applicative knowledge necessary for successful dealing with the competitive world. This year's contributions and prize winners have been selected from among a number of good, some of them even excellent works.

On behalf of the Scientific Board
Prof. Jože Drinovec

Krkine nagrade za posebne dosežke na področju raziskovalnega dela
Krka Prizes for Special Achievements in Research

Predavanja
Winners' Presentations

NAČRTOVANJE IN SINTEZA MODULATORJEV INTEGRINSKIH RECEPTORJEV

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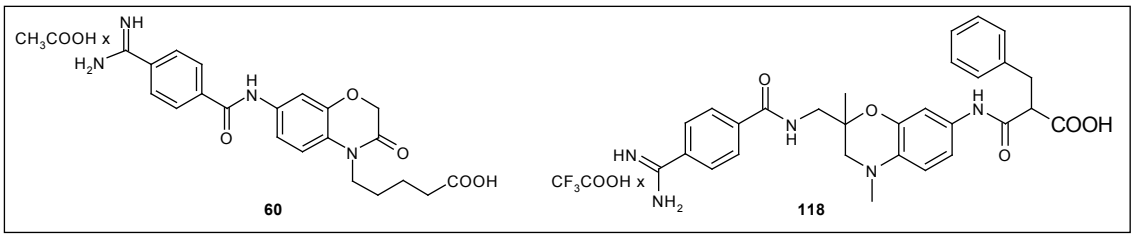
Fakulteta za farmacijo, Univerza v Ljubljani
Mentorica: M. Sollner Dolenc
Fakulteta za farmacijo, Univerza v Ljubljani

Integrini so transmembranski heterodimerni receptorji, udeleženi v interakcijah med celicami ter med celicami in zunajceličnimi proteini. Najbolj raziskana med njimi sta integrina $\alpha_{IIb}\beta_3$ (fibrinogenski receptor) in $\alpha_V\beta_3$ (vitronektinski receptor), saj sta pomembni terapevtski tarči. Integrin $\alpha_{IIb}\beta_3$ je ključni dejavnik agregacije trombocitov in zato primerna tarča za preprečevanje kardiovaskularnih in cerebrovaskularnih zapletov (npr. nestabilna angina pectoris, srčni infarkt in možganska kap), ki so posledica prekomerne agregacije trombocitov. Integrin $\alpha_V\beta_3$ omogoča adhezijo, proliferacijo in rast novih celic krvnih žil pri angiogenezi, restenozi in vnetnih obolenjih ter vgnezdenje migrirajočih rakastih celic pri tvorbi metastaz. Novejše raziskave poročajo o pomembni vlogi integrina $\alpha_V\beta_3$ pri resorpciji kosti in zato pri osteoporozi. Zadnja leta se uveljavlja koncept zaviranja delovanja $\alpha_V\beta_3$, $\alpha_V\beta_5$ in $\alpha_5\beta_1$ integrinov, saj prav vsi trije dirigirajo procese angiogeneze ter apoptoze in migracije rakastih celic (metastaziranja). Razvoj modulatorjev integrinov - njihovih antagonistov je zato obetaven pristop k novim zdravilnim učinkovinam, saj je tako terapija cerebro/kardiovaskularnih obolenj kot terapija raka, osteoporoze in vnetnih obolenj izjemno pereč problem modernega sveta.
Integrini $\alpha_{IIb}\beta_3$, $\alpha_V\beta_3$, $\alpha_V\beta_5$ in $\alpha_5\beta_1$ prepoznavajo enako aminokislinsko zaporedje - RGD (arginil-glicil-asparaginsko kislino) pri vezavi fizioloških ligandov. Omenjeno zaporedje nam je zato predstavljalo izhodišče za načrtovanje nizkomolekularnih spojin z visoko afiniteto do integrinov - modulatorjev integrinov. V okviru doktorskega dela smo uspeli pripraviti nove modulatorje integrinov na osnovi 8-amino-6-metil-2-kromankarboksilnega, 7-amino-2*H*-1,4-benzoksazin-3(4*H*)-onskega, 7-amino-2-aminometil-2,4-dimetil-3,4-dihidro-2*H*-1,4-benzoksazinskega in 7-amino-5-fenil-1,3-dihidro-2*H*-1,4-benzodiazepin-2-onskega ogrodja na katere smo pripenjali različne mimetike stranskih verig argininskega in aspartatnega dela spojine vodnice-RGD tripeptida. Osnovna ogrodja smo večinoma izbrali na osnovi znanih sinteznih postopkov[1, 2], medtem ko smo 7-amino-2-aminometil-2,4-dimetil-3,4-dihidro-2*H*-1,4-benzoksazinsko ogrodje sintetizirali po inovativni poti. Ogrodja smo uporabili kot toge mimetike osrednjega dela RGD tripeptida z namenom vzpostavljanja različnih prostorskih odnosov med mimetiki stranskih verig arginina in aspartata. S tem smo preiskovali vezavna mesta posameznih integrinov ter iskali konformacije odgovorne za vezavo in selektivnost.
Pripravljenim spojinam smo ovrednotili biološko aktivnost. Vpeljali smo testni sistem za in vitro določanje afinitete modulatorjev integrinskih receptorjev do $\alpha_{IIb}\beta_3$ in $\alpha_V\beta_3$ integrinov. Testni sistem smo ustrezno modificirali za določanje afinitete do $\alpha_V\beta_5$ in $\alpha_5\beta_1$ integrinov. Pripravljenim spojinam smo določili tudi antiagregatorno delovanje ter za spojino z največjo afiniteto do $\alpha_V\beta_3$ integrina določili njeno intrinzično aktivnost in potencialno terapevtsko uporabnost pri inhibiciji angiogeneze.

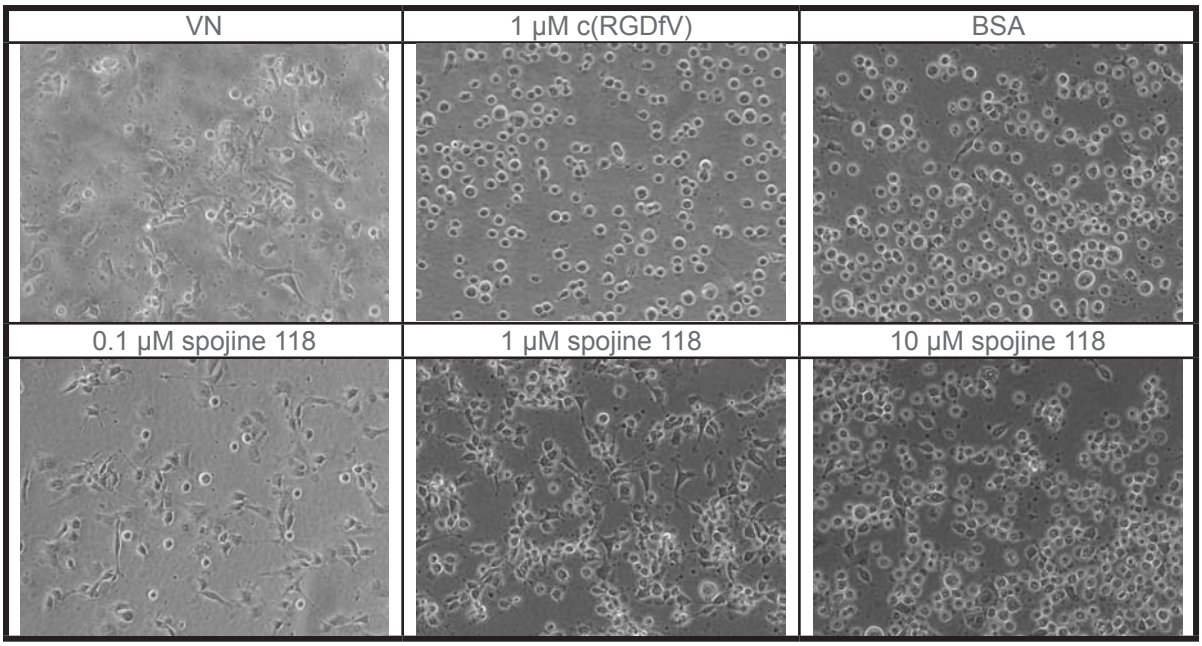


Slika 1. Spojini **60** in **118**, katerih biološki učinki izstopajo iz serij analognih spojin.

Figure 1. Compounds **60** and **118**; the most potent compounds in the series of synthesized analogues.

Rezultati testiranj kažejo, da smo pripravili močne in selektivne modulatorje $\alpha_{IIb}\beta_3$ integrina-fibrinogenskega receptorja. Spojine smo ovrednotili z uveljavljenim prostorskim modelom in ugotovili ustreznost le-tega pri načrtovanju novih modulatorjev $\alpha_{IIb}\beta_3$ integrina.[3] Ugotovili smo, da je za vezavo na $\alpha_{IIb}\beta_3$ integrin optimalna razmeroma ukrivljena konformacija spojin z močno bazičnim 4-amidinobenzoilnim fragmentom kot mimetikom

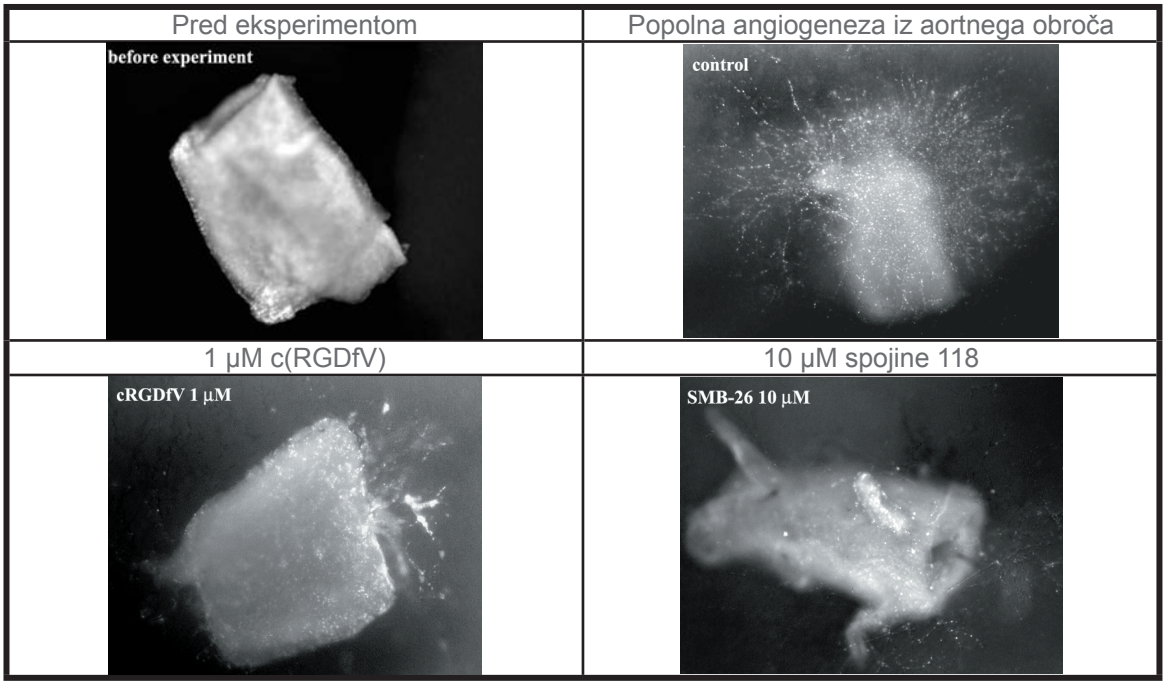
stranske verige arginina. Razdalja med kationskim in anionskim centrom se je, poleg konformacije ogrodja, izkazala za ključen dejavnik pri doseganju visoke afinitete. Pri tej skupini posebej izstopa spojina **60** s 7-amino-2H-1,4-benzoksazin-3(4H)-onskim skeletom (slika 1.), ki je kazala visoko afiniteto do $\alpha_{IIb}\beta_3$ integrina primerljivo z orbofibanom - antagonistom $\alpha_{IIb}\beta_3$ integrina.[4] Spojina **60** je kazala tudi močno, približno 100-krat močnejše antiagregatorno delovanje od referenčnega tetrapeptida RGDS, zato predstavlja spojino vodnico za načrtovanje novih, visokoafinitetnih antagonistov $\alpha_{IIb}\beta_3$ integrina.[5]



Slika 2. Vezava HUVEC celic na vitronektin (zgoraj levo). c(RGDfV) v 1 μM koncentraciji prepreči vezavo celic na vitronektin (zgoraj v sredini). Celice se ne vežejo na BSA (zgoraj desno). Spojina **118** zavira vezavo HUVEC celic na vitronektin (spodaj); pri 10 μM je inhibicija praktično popolna.

Figure 2. Binding of HUVEC cells on vitronectin (top left). c(RGDfV) in 1 μM concentration inhibits binding (top middle). HUVEC cells do not bind to BSA (top right). Compound **118** inhibits binding HUVEC cells to vitronectin (bottom); at 10 μM the inhibition is practically complete.

Selektivnost delovanja na integrin $\alpha_V\beta_3$ smo študirali in dosegli z variacijami kationskega centra s pripajanjem manj bazičnih in/ali s strani usmerjenih mimetikov stranske verige arginina. V nasprotju z literaturnimi podatki smo pokazali, da selektivnost spojin napram $\alpha_{IIb}\beta_3$ integrinu ni bistveno odvisna od razdalje med kationskim in anionskim centrom, temveč je večjega pomena usmerjenost kationskega centra ter njegov bazično-lipofilni značaj. Ugotovili smo ugoden vpliv aromatskega substituenta pripetega na α- oz. β-mesto glede na karboksilat na afiniteto do $\alpha_V\beta_3$ integrina. Pri pripravljenih modulatorjih $\alpha_V\beta_3$ integrina izstopa spojina **118** (slika 1.), ki je pokazala visoko afiniteto tudi do $\alpha_5\beta_1$ integrina in zmerno afiniteto do $\alpha_V\beta_5$ integrina. Dokazali smo, da spojina **118** deluje kot antagonist integrinske funkcije, saj zavira nekatere celične poti signalizacije, preprečuje vezavo HUVEC celic na vitronektin (slika 2.) ter deluje kot inhibitor angiogeneze (slika 3.). Jakost delovanja v funkcijskih testih je približno za en velikostni razred slabša od cikličnega pentapeptida c(RGDfV), ki je strukturni analog cilengitida - spojine v II fazi kliničnih testiranj.[6] Bistvena prednost spojine **118** pred cilengitidom je, kljub nižji jakosti delovanja, nepeptidna narava in zato možnost peroralne aplikacije. Zaradi tega predstavlja spojina **118** ugodno osnovo za načrtovanje novih, še močnejših peroralno uporabnih trojnih antagonistov integrinov.



Slika 3. Razrast novih krvnih žil iz aortnih obročev, potopljenih v matrigel. Zgoraj levo je prikazan obroč pred eksperimentom, ostali pa po sedmih dneh. Pri c(RGDfV) v 1µM je vidno zaviranje rasti novih žil (angiogeneze) iz aortnega obroča. Navidez enak ali celo boljši rezultat pri zaviranju angiogeneze je opazen pri inkubaciji s spojino **118** (SMB-26) v 10 µM koncentraciji.
Figure 3. Growth of new blood vessels out of the aortic ring submerged in the matrigel. Top left is the aortic ring before experiment; other pictures were made after 7 days of incubation. c(RGDfV) in 1µM concentration inhibits growth of new blood vessels (angiogenesis) from aortic ring. The same or even better inhibition of angiogenesis was noticed after incubation with compound **118** in 10 µM concentration.

DESIGN AND SYNTHESIS OF INTEGRIN RECEPTORS MODULATORS

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Integrins are heterodimeric transmembrane receptors involved in cell-cell and cell - extracellular matrix interactions. Among them, platelet integrin $\alpha_{IIb}\beta_3$ (fibrinogen receptor) and integrin $\alpha_V\beta_3$ (vitronectin receptor) are the best studied ones, as they are valid therapeutic targets. Integrin $\alpha_{IIb}\beta_3$ is a key factor of platelet aggregation and therefore suitable target for prevention of cardiovascular and cerebrovascular disorders (unstable angina pectoris, myocardial infarction, cerebral infarction). Integrin $\alpha_V\beta_3$ is involved in adhesion, proliferation and growth of vascular cells during angiogenesis, restenosis and inflammatory processes. It enables the embedding and spreading of tumour cells and osteoclasts during metastasis and bone resorption. Recently, the concept of multiple $\alpha_V\beta_3$, $\alpha_V\beta_5$ and $\alpha_5\beta_1$ integrin antagonism has emerged, as all of them regulate promotion of angiogenesis, apoptosis and migration of tumour cells. The development of integrin modulators-their antagonist is therefore a promising approach to new drugs, which are of significant importance in the growing therapeutic area of cerebro/cardiovascular disorders, cancer, osteoporosis and inflammatory processes.

$\alpha_{IIb}\beta_3$, $\alpha_V\beta_3$, $\alpha_V\beta_5$ in $\alpha_5\beta_1$ integrins share the same binding amino acid sequence of the native ligands-RGD sequence (arginyl-glycyl-aspartic acid). This sequence was identified as a lead structure for the design of low-molecular compounds with high affinity to integrins-the integrins modulators. We have designed integrin modulators on the basis of variously substituted 8-amino-6-methyl-2-cromanecarboxylates, 7-amino-2*H*-1,4-benzoxazine-3(4*H*)-ones, 7-amino-2-aminomethyl-2,4-dimethyl-3,4-dihydro-2*H*-1,4-benzoxazines and 7-amino-5-phenyl-1,3-dihydro-2*H*-1,4-benzodiazepine-2-ones. These starting scaffolds were selected mostly on the basis of previously described synthetic procedures[1, 2], while the 7-amino-2-aminomethyl-2,4-dimethyl-3,4-dihydro-2*H*-1,4-benzoxazine was synthesized with innovative procedure. The scaffolds were employed as rigid mimetics of the central part of RGD tripeptide in order to achieve different spatial orientations between mimetics of arginine and aspartate side chain functionalities. Thus we planned to explore the binding sites of individual integrins. Resulting compounds with defined conformations were designed to elucidate structural features required for high affinity and selectivity towards various integrins.

Compounds synthesized were evaluated in various biological assays. We have used the in vitro assay for the measurement of binding affinities for $\alpha_{IIb}\beta_3$ and $\alpha_V\beta_3$ integrins. The assay was modified in order to determine binding affinities for $\alpha_V\beta_5$ and $\alpha_5\beta_1$ integrins. The antiaggregatory activities of the synthesized compounds were assessed as well. Compound with the highest affinity for $\alpha_V\beta_3$ integrin was characterized furthermore in order to determine it's intrinsic activity and therapeutic potential as angiogenesis inhibitor.

The results of biological assays indicate that some of the prepared compounds are potent and selective modulators of $\alpha_{IIb}\beta_3$ integrin. These compounds fit the proposed 3D-binding model demonstrating its validity in the design of new modulators of $\alpha_{IIb}\beta_3$ integrin.[3] We have established that the binding conformation that suits the most the binding site of $\alpha_{IIb}\beta_3$ integrin is a slightly curved one with strong basic 4-amidinobenzoyl fragment as an arginine side chain mimetic. The distance between cationic and anionic centre of the molecule was found out to be a key feature for high affinity. The compound **60** with 7-amino-2*H*-1,4-benzoxazin-3(4*H*)-one scaffold (figure 1.) has shown high affinity towards $\alpha_{IIb}\beta_3$ integrin which was comparable to the orbofiban – the antagonist of $\alpha_{IIb}\beta_3$ integrin.[4] This compound also possesses 1000-fold more potent antiaggregatory activity than the reference tetrapeptide RGDS thus revealing its potential as a lead compound in the design of new high-affinity $\alpha_{IIb}\beta_3$ integrin antagonists.[5]

The selectivity towards $\alpha_V\beta_3$ integrin was studied and achieved through variations of cationic centre with the use of less basic and "side-on" oriented arginine side chain mimetics. In the contrast with the published results, we have clearly demonstrated that the selectivity versus $\alpha_{IIb}\beta_3$ integrin does not depend mostly on the distance between cationic and anionic centre, but upon the orientation and basic-lipophylic character of the cationic centre. We have also established that aromatic substituents attached to α- or β-position relative to carboxylate moiety may contribute to high affinity for $\alpha_V\beta_3$ integrin. Compound **118** showed high affinity towards $\alpha_V\beta_3$ and $\alpha_5\beta_1$ integrins and modest affinity towards $\alpha_V\beta_5$ integrin (figure 1.). We have proved that compound **118** acts as an antagonist of integrin function as it inhibits some cell signalisation pathways, it inhibits binding of HUVEC cells on vitronectin (figure 2.) and acts as angiogenesis inhibitor (figure 3.). Its potency in function assays is determined to be approximately one order of magnitude lower than that of cyclic pentapeptide c(RGDfV), which is structural analogue of cilengitide – the compound in the phase II of clinical trials.[6] The essential advantage of **118** over the c(RGDfV) is its non-peptide structure, which should enable oral application of the compound. Therefore, compound **118** may be regarded as an excellent basis for the development of new, more potent orally active triple integrin antagonists.

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ELEKTROFILNO AMINIRANJE NEKATERIH SUBSTRATOV Z RAZLIČNIMI DIAZENI

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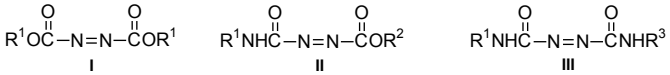
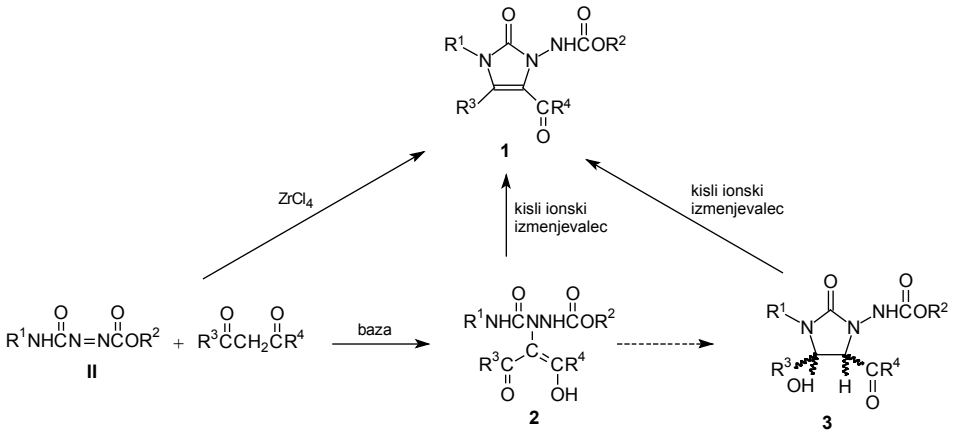
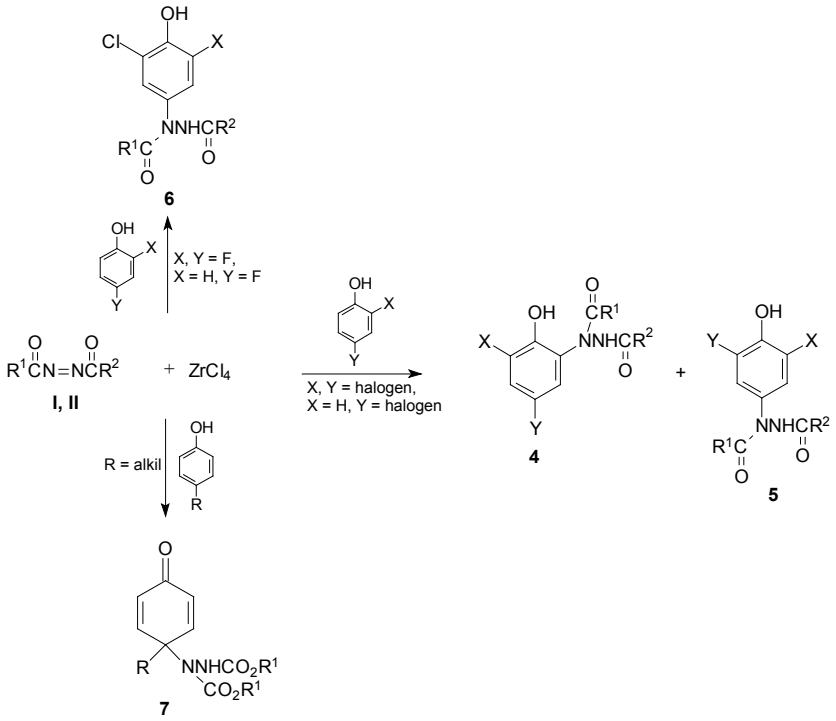
Dušik je eden najpomembnejših elementov v organski kemiji, saj je zastopan v številnih biološko aktivnih spojinah. Obstaja veliko metod za uvedbo dušika v obliki funkcionalne skupine v molekulo, vendar pa večina teh zahteva ostre reakcijske pogoje (npr. močno kisli medij, visoke temperature,...). Pri načrtovanju organskih sintez so izrednega pomena reagenti, s pomočjo katerih lahko uvedemo dušik v molekulo. Eni izmed njih so tudi diazeni; to so spojine, ki imajo v svoji strukturi N=N skupino, katere dušika sta elektrofilna, in so zato primerni reagenti za elektrofilno aminiranje.
Disertacija obravnava raziskovanje lastnosti in uporabo diazenov **I**, **II** in **III** kot reagentov za elektrofilno aminiranje različnih substratov.



Sintetizirala sem nekatere nove alkil aminokarbonildiazenkarboksilate **II** in jih pretvorila v ustrezne diazendikarboksamide **III**. Testiranja določenih tipov diazenov so pokazala visoko citotoksično aktivnost na različne tumorne celice.
V prvem delu raziskav sem preučevala aminiranje različnih spojin z aktivirano metilensko skupino (1,3-diketoni, β-ketoestri in β-ketoamidi) v prisotnosti $ZrCl_4$ kot katalizatorja. Pri tej reakciji so nastali imidazolin-2-oni **1** z visokimi izkoristki. Spojine **1** sem izolirala tudi, če sem kot katalizator uporabila trifluoroocetno kislino. Po drugi strani pa so pri bazično kataliziranem aminiranju izbranih 1,3-dikarbonilnih spojin nastali Michaelovi adukti **2** in v nekaterih primerih tudi 2-imidazolidinoni **3**, pri čemer sem kot bazo uporabila 1,5-diazabiciklo[3.4.0]non-5-en (DBN) ali natrijev acetat. Omenjene reakcije so potekle regioselektivno, tako glede na diazen, kakor tudi na 1,3-dikarbonilno spojino. Tako je v vseh primerih reagiral diazenski dušik ob amidni skupini, ciklizacija do končnih produktov **1** pa je v primeru nesimetričnih 1,3-dikarbonilov potekla z bolj reaktivno acilno skupino. Michaelove produkte **2** in 2-imidazolidinone **3** sem v prisotnosti kislega ionskega izmenjevalca pretvorila v imidazolin-2-one **1**, ki so nastali kot edini produkti pri aminiranju kataliziranem s $ZrCl_4$. S tem sem potrdila tezo, da so Michaelovi adukti **2** in 2-imidazolidinoni **3** res intermediati pri nastanku imidazolin-2-onov **1** iz diazenov **II** in 1,3-dikarbonilov.



Nadalje sem svoje raziskovanje osredotočila na elektrofilno aromatsko aminiranje. Aktivirane aromate sem uspešno aminirala na njihovem najreaktivnejšem mestu. Reakcije so potekle tudi v tem primeru regioselektivno glede na diazen. Čeprav prisotnost halogena deaktivira aromatski obroč za elektrofilne reakcije, so 2,4-dihalogenirani in 4-monohalogenirani fenoli reagirali z diazendikarboksilati **I** in aminokarbonildiazenkarboksilati **II** pod izredno milimi reakcijskimi pogoji. Opazila sem, da poleg pričakovanega produkta **4**, nastane še njegov *para*-aminiran izomer **5**, kot posledica procesa, pri katerem med aminiranjem halogen (fluor, klor, brom in jod) migrira po aromatskem obroču s *para* na *orto* mesto glede na fenolno OH skupino. V nekaterih primerih je prišlo do odstranitve halogena z aromatskega obroča ali pa do zamenjave s klorovim atomom iz $ZrCl_4$. 4-Hidrazino-2-klorofenoli **6**, ki sem jih izolirala ko edine produkte pri reakciji 4-fluorofenola z različnimi diazeni **I** in **II**, so najverjetneje nastali tako, da je med aminiranjem potekla še substitucija fluora s klorom. Premestitev halogenov pa nisem opazila pri reakciji 2-halofenolov, kjer je aminiranje poteklo na para mestu glede na OH skupino. Čeprav so podobne reakcije aminiranja haloarenov z diazendikarboksilati **I** znane, pa premestitve halogenov (halogen dance) tekom elektrofilnega aminiranja še niso bile opisane.



Pri iskanju mehanizma aminiranja halofenolov z diazeni v prisotnosti $ZrCl_4$ so bili ključnega pomena produkti aminiranja 4-alkilfenolov. Pri tej reakciji so nastali 2,5-cikloheksadienoni **7**, ker migracija alkilne skupine ni možna. Ti rezultati so potrdili predlagan mehanizem elektrofilnega aromatskega aminiranja različnih halofenolov, med katerim poteka t.i »halogen dance«.

Ugotovila sem, da $ZrCl_4$ ni mogoče zamenjati s ZrF_4 ali $ZrBr_4$, čeprav lahko slednjega uporabimo skupaj z diazendikarboksilatom v ekvimolarnih količinah kot učinkovito sredstvo za regioselektivno bromiranje različnih aromatskih in heteroaromatskih spojin.

Rezultati kažejo kako široko uporabna skupina reagentov za aminiranje so diazeni. Predvsem aminokarbo nildiazenkarboksilati **II** so zaradi različnih reaktivnih centrov v svoji strukturi pomembni za sintezo številnih organskih spojin (imidazol-2-onov, 2-imidazolidinonov, aromatskih in alifatskih hidrazinkarboksilatov). Poleg tega so nekateri diazeni tudi biološko aktivni, kar še dodatno veča zanimanje za raziskovanje teh spojin.

ELECTROPHILIC AMINATION OF VARIOUS SUBSTRATES WITH DIFFERENT DIAZENES

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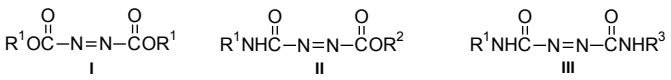
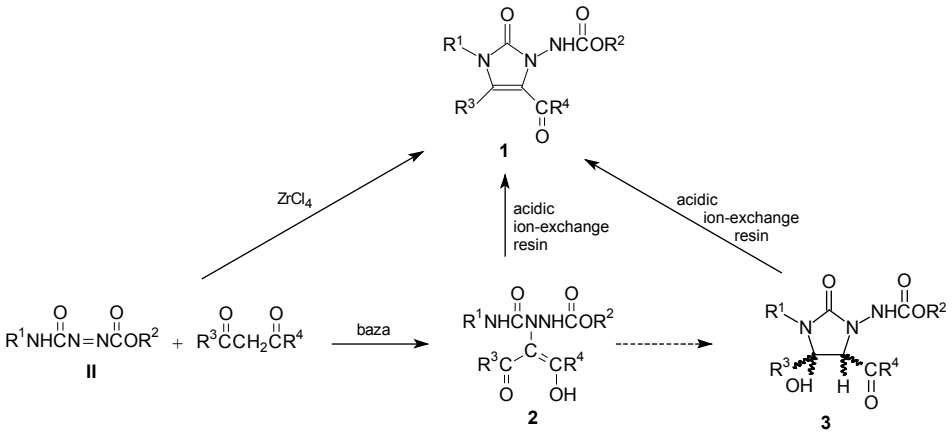
The nitrogen is one of the most important elements in organic chemistry as it is incorporated in almost all classes of biologically active molecules. Conventional methods for the synthesis of amino derivates usually demand harsh reaction conditions (high temperatures, strong acid media,…). It has been demonstrated that diazenes are an appropriate source of electrophilic nitrogen and thus consequently useful reagents for aminations.



The Thesis describes features and applications of diazenes **I**, **II** and **III** as reagents for the amination of various substrates.

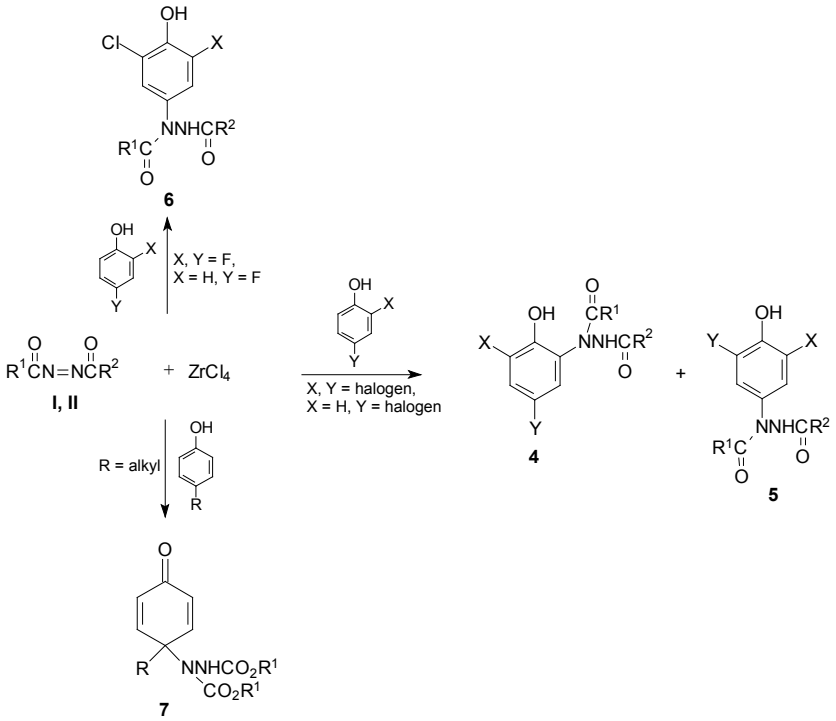
In the first part of my research work new alkyl aminocarbonyldiazenecarboxylates **II** were synthesized and transformed to the corresponding diazenedicarboxamides **III**. Some of diazenes have been found to exhibit cytotoxic activity against various tumour cell lines *in vitro*.
Electrophilic aminations of compounds which possess an activated methylene group (1,3-diketones, β-ketoesters and β-ketoamides) were performed in the presence of $ZrCl_4$ as a catalyst. Diazenes reacted under mild reaction conditions to give highly functionalized imidazolin-2-ones **1** in excellent yields. Reactions took place with complete regioselectivity concerning both partners, the diazene and the 1,3-dicarbonyl. Accordingly, diazenes reacted allways with the nitrogen vicinal to the amide functionality. The reaction is followed by the ring closure with more reactive acyl group originating from the 1,3-dicarbonyle. The same products **1** were obtained when trifluoroacetic acid was used as a catalyst. The base-catalysed aminations of selected 1,3-dicarbonyles resulted in the formation of Michael-type adducts **2** or in some cases, 2-imidazolidinones **3**. The reactions proceeded again regioselectively. The compounds obtained under basic conditions have been transformed to the imidazolin-2-ones **1** using a strongly acidic ion-exchange resin.



The second type of reactions I examined involved electrophilic aromatic aminations of activated arenes in the presence of $ZrCl_4$. In these reactions aromatic hydrazides were formed and only one regioisomer was observed. Reaction took place regioselectively in regard to both partners; thus arenes reacted at the most activated position with electrophilic nitrogen vicinal to the amide group of the diazenes **II**.

Although a halogen substituent deactivates aromatic ring for electrophilic substitutions, 2,4-dihalo- and 4-halophenols reacted with diazenes **I** and **II** under mild reaction conditions in the presence of $ZrCl_4$ to afford a mixture of regioisomeric products **4** and **5**. The compounds **4** can be considered as a »normal« products of amination. On the other hand, the regioisomers **5** can be formed in the process that involves the migration of one of the halogen atoms from the starting 2,4-dihalophenol. During amination a halogen (fluorine, chlorine, bromine and iodine) migrated from *para* to *ortho* position with regard to the phenolic OH or was substituted with the chlorine atom originating from $ZrCl_4$. By performing an electrophilic aromatic substitution on 2-halophenols, the hydrazino moiety was completely directed to the *para* position Described reactions represent the first example of »halogen dance« during the electrophilic amination promoted by $ZrCl_4$.

To get a better insight into the mechanism of electrophilic aromatic amination we performed some additional experiments on *para*-alkyl substituded phenols. 2,5-Cyclohexadienones **7** were isolated in excellent yields after 4-alkylphenols were treated with diazenes using $ZrCl_4$ as Lewis acid, since an alkyl group can not migrate during amination. On the basis of performed experiments a plausible reaction pathway was proposed for the amination of halophenols with diazenes.



$ZrBr_4$ as well as ZrF_4 can not be used instead of $ZrCl_4$ in above mentioned reactions. When a system $ZrBr_4$/ dialkyl diazenedicarboxylate was employed various aromatic and heteroaromatic compounds have been successfully brominated at the most activated position.

The results showed that diazenes are very efficent aminating reagents in organic chemistry. Since aminocarbonyldiazenecarboxylates **II** posses several reactive centers they may serve as building blocks for other biologically important molecules (imidazol-2-ones, 2-imidazolidinones, aliphatic and aromatic hydrazinecarboxylates).

IZDELAVA PELET Z RAZLIČNIMI GRANULACIJSKIMI TEKOČINAMI IN VPLIV HIDRODINAMSKIH RAZMER V WURSTERJEVI KOMORI NA UČINKOVITOST FILMSKEGA OBLAGANJA

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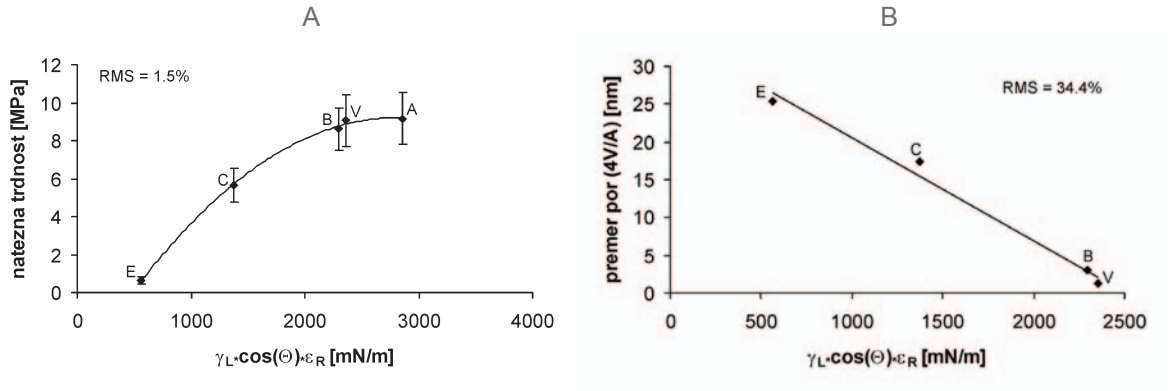
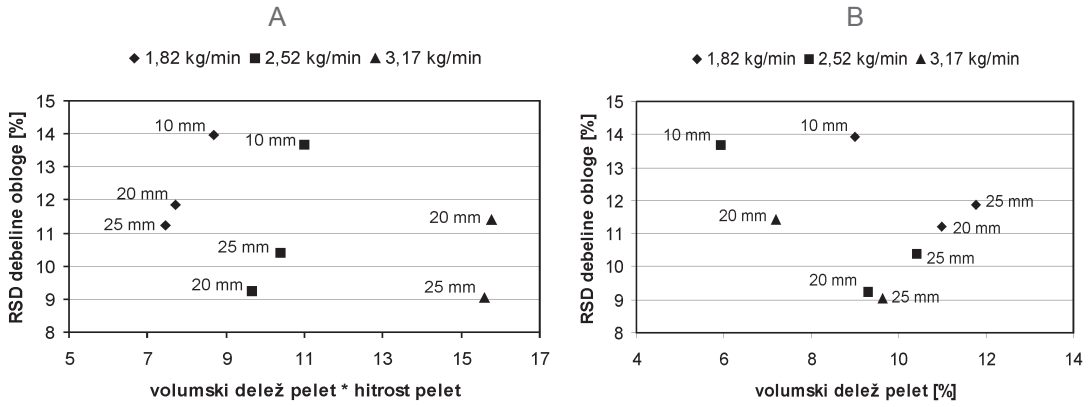
Razumevanje fizikalnih procesov, ki potekajo in so prisotni med delci trdne snovi ter tekočino, je pomembno pri nadzorovanju tehnološkega procesa in izdelovanju farmacevtske oblike. Namen dela je določiti vpliv granulacijske tekočine na lastnosti pelet ter poiskati ustrezno teoretično razlago oz. fizikalno količino, ki bi opisovala spremembe lastnosti pelet preko razlik v lastnostih uporabljenih granulacijskih tekočin. Uporaba etanola ali vodno/etanolnih mešanic kot granulacijskih tekočin v postopku iztiskanja in kroglicenja rezultira v nastanku pelet z bistveno drugačnimi mehanskimi in strukturnimi lastnostmi kot v primeru, ko kot granulacijsko tekočino uporabimo vodo. Vpeljali smo zmnožek površinske napetosti (γ_L), dielektrične konstante granulacijske tekočine (ε_R) in kosinusa stičnega kota granulacijske tekočine s trdnimi snovmi pelete ($\cos(\Theta)$) z namenom razlage mehanizma nastanka razlik v lastnostih pelet. Produkt $\gamma_L \cdot \cos(\Theta) \cdot \varepsilon_R$ smo predlagali, ker menimo, da obstaja fizikalno ozadje, ki omogoča razlago vpeljanega zmnožka kot sorazmernega faktorja s seštevkom sil kontrakcije aglomerata med sušenjem ter sil, ki zgostitvi aglomerata nasprotujejo.
Za opis medfaznih pojavov tekoče - trdno je potrebno poznavanje polarnih in disperzijskih prispevkov proste površinske energije snovi. Prispevke prostih površinskih energij za trdne snovi pelet in granulacijske tekočine smo določili z merjenjem stičnega kota z Wilhelmijevo metodo s ploščico. Pelete smo izdelali s tehnologijo iztiskanja in kroglicenja ob uporabi vode, etanola in vodno/etanolnih mešanic kot granulacijskih tekočin. Zaradi lažje razlage povezav med vpeljanim zmnožkom in lastnostmi pelet smo pelete izdelali le z uporabo mikrokristalne celuloze kot trdne snovi.
Vrednotenje izdelanih pelet je pokazalo, da natezna trdnost (slika 1A) in čas razpadnosti pelet naraščata z naraščanjem vrednosti vpeljanega zmnožka $\gamma_L \cdot \cos(\Theta) \cdot \varepsilon_R$, medtem ko se obrabnost, povprečni premer por (slika 1B) in poroznost pelet zmanjšujejo.



Slika 1: Natezna trdnost (A) in povprečni premer por (B) vzorcev pelet, izdelanih z uporabo različnih granulacijskih tekočin, kot funkcija vpeljanega zmnožka; V – prečiščena voda, E – brezvodni etanol, A – etanol/voda (4,9 mol%), B - etanol/voda (8,2 mol%), C – etanol/voda (21,1 mol%)

Obstoj korelacij med predlaganim zmnožkom in lastnostmi pelet kaže na to, da granulacijska tekočina vpliva na končne mehanske in strukturne lastnosti pelet prek vpliva na velikost sil kontrakcije in sil, ki zgostitvi med procesom sušenja aglomerata nasprotujejo. Hkrati pa poznavanje vrednosti predlaganega zmnožka nakazuje možnost napovedovanja mehanskih lastnosti pelet. Tako pelete, izdelane z uporabo 4,9 mol% etanolno -vodne mešanice, izkazujejo boljše mehanske lastnosti, kot tiste, izdelane z vodo, kar napovedujeta že vrednosti zmnožkov za obe granulacijski tekočini.

Poznavanje, razumevanje in obvladovanje postopka oblaganja z Wursterjevo procesno komoro nam omogoča zagotavljanje zveznih, enakomernih in ponovljivih filmskih oblog ob maksimalnem izkoristku disperzije za oblaganje. Namen dela je pokazati povezavo med enakomernostjo debeline obloge od pelete do pelete ter povprečnim volumskim deležem pelet v področju razmejitvenega valja Wursterjeve komore in s tem eksperimentalno potrditi pomen volumskega deleža pelet.
V ta namen smo obložili vzorce pelet pri različnih masnih tokovih zraka in različnih velikostih reže med distribucijsko ploščo in razmejitvenim valjem. RSD-je debelin filmske obloge smo določili s posredno metodo določanja raztrosa debeline filmske obloge z določanjem koncentracije barvila v filmski oblogi. Pri enakih nastavitvah komore in tehnološkega procesa kot v primeru eksperimentov oblaganja smo z vgradnjo lopute v komoro določili volumske deleže pelet v področju razmejitvenga valja. Z množenjem volumskega deleža pelet in lokalne hitrosti pelet, ki smo jo določili s snemanjem s hitro kamero, smo dobili relativno merilo masnega toka pelet.
Ugotovili smo, da je masni tok pelet pri določenem masnem toku zraka skozi komoro skoraj konstanten oziroma neodvisen od velikosti reže med razmejitvenim valjem in distribucijsko ploščo (slika 2A). Primerjava med merilom za masni tok pelet (ε_S*hitrost pelet) ter RSD-jem debeline obloge ni pokazala povezave, medtem ko se RSD debeline filmske obloge linearno povečuje z večanjem volumskega deleža pelet v področju oblaganja (ε_S) kot posledica učinka senčenja, vendar le za eksperimente oblaganja s podobnimi velikostmi rež med razmejitvenim valjem in distribucijsko ploščo (20, 25 mm) (slika 2B). Menimo, da so višje vrednosti RSD-ja debeline filmske obloge za vzorce pelet, ki so bili obloženi pri 10 mm reži med razmejitvenim valjem in distribucijsko ploščo, posledica manj enakomernega kroženja pelet med postopkom oblaganja v primerjavi z oblaganji pri večjih velikostih reže.



Slika 2: Raztros debeline filmske obloge kot funkcija relativnega masnega toka pelet (A) in volumskega deleža pelet (B)

Z namenom razumevanja rezultatov in želje po nadomestitvi eksperimentov smo pričeli z razvojem CFD simulacije gibanja delcev v Wursterjevi komori. S Pitotovo cevjo smo pomerili hitrostne profile zraka znotraj razmejitvenega valja Wursterjeve komore ter izračunali masne tokove zraka. S CFD orodjem Fluent 6.1 smo razvili simulacijo gibanja toka zraka skozi Wursterjevo komoro ob odsotnosti pelet. Na podlagi primerjave eksperimentalnih vrednosti masnih tokov zraka skozi razmejitveni valj s simulacijskimi vrednostmi ter linearnosti rešitev simulacije lahko trdimo, da simulacija ob načrtani računski mreži, izbranem standardnem k-ε turbulentnem modelu ter predpisanih robnih pogojih daje ustrezne rezultate. Dobljeni rezultati predstavljajo osnovo za nadaljnji razvoj simulacije, ki bi vključevala tok trdnih delcev.

PRODUCTION OF PELLETS WITH DIFFERENT GRANULATION LIQUIDS AND INFLUENCE OF HYDRODYNAMIC CONDITIONS IN WURSTER CHAMBER ON EFFICIENCY OF PELLET FILM COATING

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Understanding of the physical processes, which are occurring and are present at the solid – liquid interface, is important factor in controlling the technological process of the dosage form production. The purpose of this work is to assess the influence of granulating liquid on the properties of pellets and to find an adequate theoretical explanation or physical quantity which would describe change of pellet properties as function of change in physico-chemical properties of used granulating liquid. The use of ethanol or ethanol/water mixtures as granulation liquids in the extrusion-spheronisation process results in the formation of pellets with significantly different mechanical and structural properties from those prepared using of water alone. The product of surface tension (γ_L), relative permittivity (ε_R) of the granulation liquid and the cosine of contact angle (Θ) of granulation liquid on pellets solid has been introduced in order to explain the mechanism of this phenomenon. We have introduced the $\gamma_L \cdot \cos(\Theta) \cdot \varepsilon_R$ which can be considered to represent the driving and counteracting forces of pellet contraction during drying.
The contact angles and surface tensions were evaluated using the Wilhelmy plate method. Pellets were produced by extrusion-spheronisation technique using water, ethanol and ethanol/water mixtures as granulation liquids.
Subsequent characterization of the pellets showed that their tensile strength (Fig. 1A) and disintegration times increase with increase in the proposed factor $\gamma_L \cdot \cos(\Theta) \cdot \varepsilon_R$, while friability, average pore diameter (Fig. 1B) and porosity decreases.

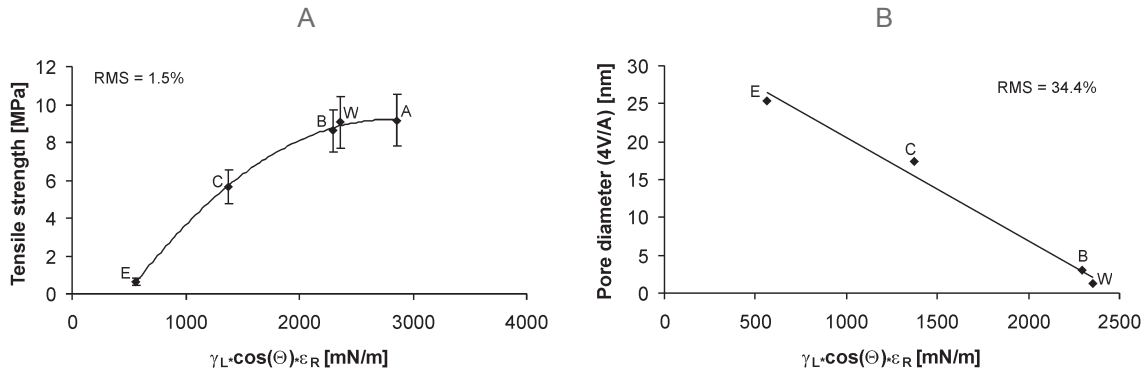

Fig. 1: Tensile strength (A) and mean pore diameter (B) of pellets prepared with different granulation liquids as function of $\gamma_L \cdot \cos(\Theta) \cdot \varepsilon_R$; W – water; E – ethanol; A – ethanol/water (4.9 mol%); B - ethanol/water (8.2 mol%); C – ethanol/water (21.1 mol%).

The observed correlations show that the granulation liquid influences the mechanical and structural properties of the pellets through the contraction driving and contraction counteracting forces during drying. Knowledge of the value of the introduced product for certain granulating liquid could be used in prediction of mechanical properties of pellets. This can be confirmed by the values of the product for 4.9 mol% ethanol/water mixture and purified water which predict better or at least equal mechanical properties of pellets produced by use of 4,9 mol% ethanol/water mixture in comparison with pellets produced by use of water.

Knowledge, understanding and control of the Wurster coating process enable us to assure coherent, uniform and reproducible film coatings with high utilization of the film coating dispersion. The purpose of this work is to show the relation between uniformity of the polymer film applied on pellets and pellets average volume fraction inside Wurster partition and with this to experimentally confirm the significance of the solids volume fraction inside Wurster partition.
The pellets were coated in Wurster process chamber using different air mass flow rates of the fluidizing air and different sizes of gap between distribution plate and Wurster partition. Then the RSD-s of the pellets film coating thickness were assessed by measuring the concentration of the colouring agent which was incorporated in the coating. Using the same process and geometric parameters as in coating experiments pellets average volume fractions inside Wurster partition were measured by means of the inbuilt hatch used as a valve. The relative measure for the mass flow rate of pellets was obtained by multiplying the value of the measured pellets volume fraction with the value of the average local speed of pellets, determined by the high-speed camera.
The intensity of pellets mass flow rate was shown to be independent of gap size between distribution plate and Wurster partition while using constant air mass flow rate (Fig. 2A). No correlation was found when relating measure for the pellets mass flow rate with the RSD of the film coating thickness. It has been demonstrated that the RSD of the film coating thickness increases linearly with increase of pellet volume fraction inside Wurster partition although only for coating experiments with similar gap sizes (20, 25 mm) (Fig. 2B). The linear relationship is in agreement with the shading effect proposed in the literature. It is believed that high values of the RSD of the film coating thickness, determined for film coating experiments using 10 mm gaps, are the consequence of less uniform pellet circulation during processing when compared to experiments performed with larger gap sizes.

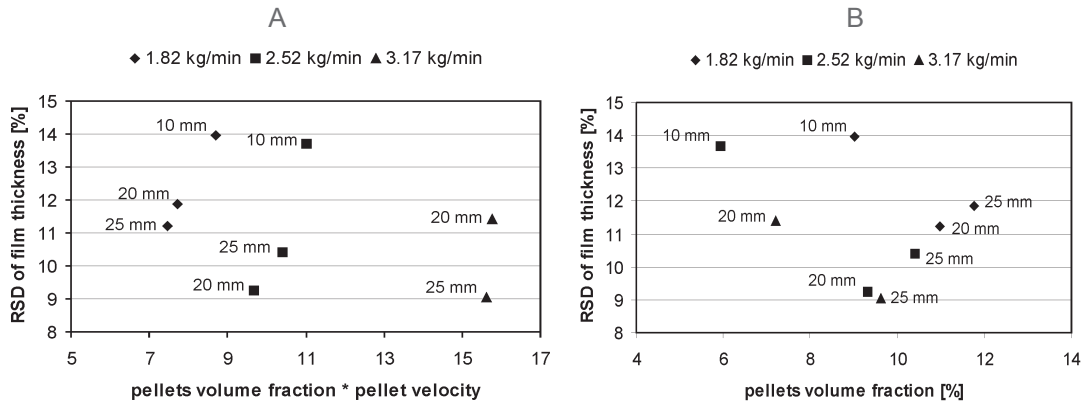

Fig. 2: RSD of the film coating thickness as function of the relative mass flow rate of pellets (A) and pellets volume fraction (B).

The development of the CFD simulation of the particles movement inside Wurster chamber was started with purpose of understanding the coating process and replacing the experiments. Velocity profiles of fluidizing air inside Wurster partition were measured using Pitot tube and then the mass flow rates were calculated. The simulation of the air flow through the Wurster chamber in absence of pellets was developed using CFD code Fluent 6.1. On basis of comparing experimental values of air mass flow rates through Wurster partition with values obtained with simulations it can be concluded that simulating using the generated mesh, selected k-ε turbulent model and prescribed boundary conditions results in adequate solution. Current development stage of the simulation represents basis for the development of the simulation that would include the solid phase flow.

STRUKTURNE RAZISKAVE PREDORGANIZACIJE PROSTORSKIH STRUKTUR OLIGOMERNIH NUKLEINSKIH KISLIN

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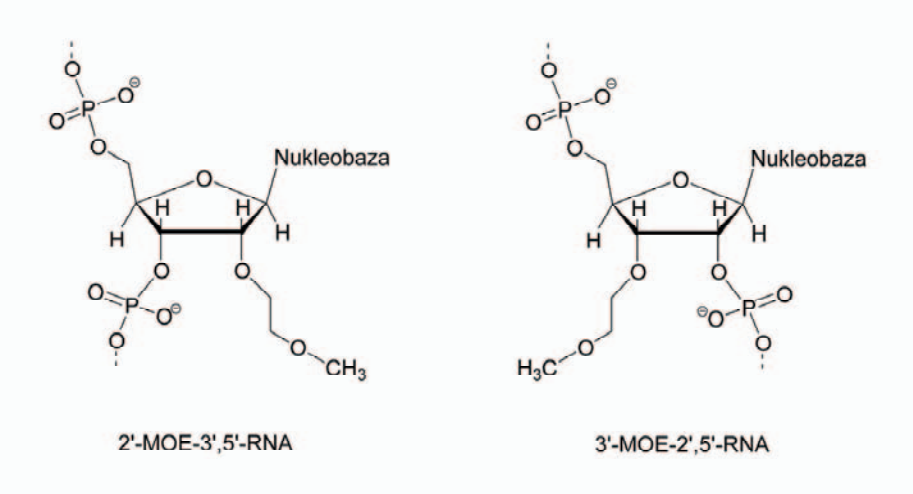
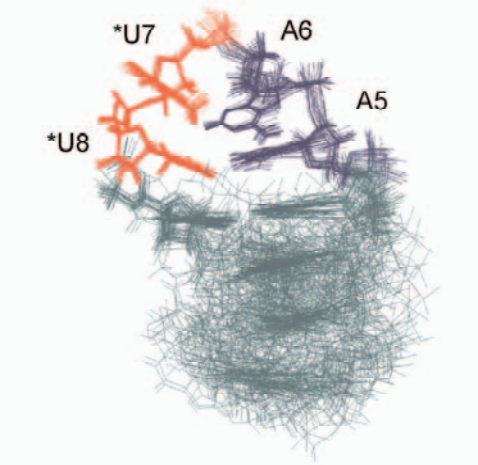
Molekule RNA in DNA tvorijo kompleksne prostorske strukture, ki sodelujejo v bioloških procesih, kot so zapis in prenos genske informacije, katalizni procesi ter interakcije z drugimi molekulami v celici. Modifikacije struktur nukleinskih kislin lahko poudarijo lastnosti, ki so že prisotne v samih nukleinskih kislinah. Še bolj pomembno pa je, da modifikacije vodijo do novih lastnosti, ki jih naravne RNA in DNA nimajo. Takšne lastnosti so zelo pomembne za uporabnost modificiranih nukleinskih kislin v medicini, biologiji, biokemiji in biotehnologiji. Poznavanje prostorskih struktur nukleinskih kislin je ključnega pomena za razumevanje njihovih bioloških funkcij.
V disertaciji sta predstavljeni prostorski strukturi dveh modificiranih oligonukleotidov in opis vpliva modifikacij sladkorno-fosfatnega skeleta na medverižno asociacijo posameznih vijačnic. Konformacijska predorganizacija oligonukleotidne verige, da tvori določeno prostorsko strukturo pred vezavo v kompleks z različnimi molekulami, je zelo odvisna od izbire substituentov na sladkorno-fosfatnem skeletu ter heterociklični nukleobazi. V ta namen smo z uporabo metode jedrske magnetne resonance (NMR) in molekulsko dinamskih simulacij (MD) raziskovali prostorsko strukturo dveh modificiranih oligonukleotidov zaporedja *CG*CGAA*U*U*CG*CG z različno fosfodiestersko povezavo med nukleotidi. Omenjeno zaporedje služi kot model eksperimentalnih in teoretičnih raziskav v strukturni biologiji nukleinskih kislin. Oba oligonukleotida imata enake funkcionalne modifikacije sladkorno-fosfatnega skeleta, vendar je pri 2',5'-povezanem oligonukleotidu metoksietilna (MOE) skupina vezana na 3'-mestu sladkornega obroča, medtem ko je pri 3',5'-povezanem oligonukleotidu vezana na 2'-mesto sladkornega obroča. Citozinske in uracilske nukleobaze so metilirane na mestu 5.



Pokazali smo, da je 3'-MOE-2',5'-RNA oligonukleotid udeležen v ravnotežju med strukturama lasnice in dupleksa, ki je odvisno od koncentracije oligonukleotida, koncentracije soli, temperature in pH raztopine. V ozkem fiziološkem območju smo opazili nenavadno pH odvisnost širjenja linij imino protonskih resonanc, ki se izmenjajo pri nižjem pH, medtem ko so pri višjem pH relativno ozke. Poleg tega pH vpliva tudi na dinamiko odpiranja in zapiranja baznih parov 2',5'-povezanega dupleksa. Širjenje linij imino resonanc smo razložili z modelom dihanja, pri katerem pride do prekinitve in tvorbe medverižnih vodikovih vezi s hitrostjo, ki je vmesna na NMR časovni skali.
Kot prvi smo v znanstveni literaturi (Plevnik et al. (2005) *Nucleic Acids Res.*, 33, 1749-1759.) opisali prostorsko strukturo lasnice, ki ima le 2',5'-fosfodiesterske vezi. Zanka 2',5'-povezane lasnice je nov strukturni motiv in je zelo dobro strukturno določena, stebelni del pa bolj fleksibilen. Običajno so stebelni deli lasnic bolj določeni in

zanke bolj fleksibilne. Zanka lasnice je poleg Watson-Crick A5•*U8 baznega para stabilizirana še z neveznimi n → π* interakcijami med prostimi elektronskimi pari O4' atomov in aromatskih obročev baz. Kakor kaže imajo nevezne n → π* interakcije pomembno vlogo pri stabilizaciji struktur z 2',5'-fosfodiestersko povezavo. Poleg tega naša raziskava potrjuje opažanja, da končni nukleotidi 2',5'-povezanih nukleinskih kislin zasedejo sin konformacijo vzdolž glikozidne vezi.



prostorske strukture 3'-MOE-2',5'-RNA lasnice

Opisali smo tudi prostorsko strukturo 2'-MOE-3',5'-RNA dupleksa (zaporedje *CG*CGAA*U*U*CG*CG[1], kjer je G[1] je 2'-deoksigvanozin), ki je analog 3'-MOE-2',5'-RNA oligonukleotida. Talilna temperatura 2'-MOE-3',5'-RNA dupleksa je zelo visoka (T_m ≈ 95°C), kar kaže na tvorbo zelo stabilnega dupleksa z močno afiniteto do tvorbe baznih parov in močnih interakcij nalaganja. S pomočjo NMR eksperimentov smo določili strukturne omejitve in s pomočjo MD simulacij izračunali prostorsko strukturo dupleksa, ki ima obliko A-tipa dvojne vijačnice. Primerjava z nemetiliranim analogom 2'-MOE RNA dupleksa je pokazala, da C5-metilacija citozinskih in uracilski baz nima večjega vpliva na prostorsko strukturo, saj so vzorci nalaganja baz v obeh dupleksih podobni.


prostorske strukture 2'-MOE-3',5'-RNA dupleksa

Naše raziskave kažejo na velik vpliv sprememb sladkorno-fosfatnega skeleta na zvitje struktur nukleinskih kislin. Modificirani oligonukleotidi so močno biološko in biokemijsko orodje protismerne tehnologije, ki uravnava izražanje in funkcijo genov in vitro in in vivo. Poznavanje modifikacij in njihovega vpliva na prostorske strukture nukleinskih kislin pripomore k načrtovanju boljših in efektivnejših terapevtskih učinkovin.

STRUCTURAL STUDIES OF PREORGANIZATION OF OLIGOMERIC NUCLEIC ACIDS STRUCTURES

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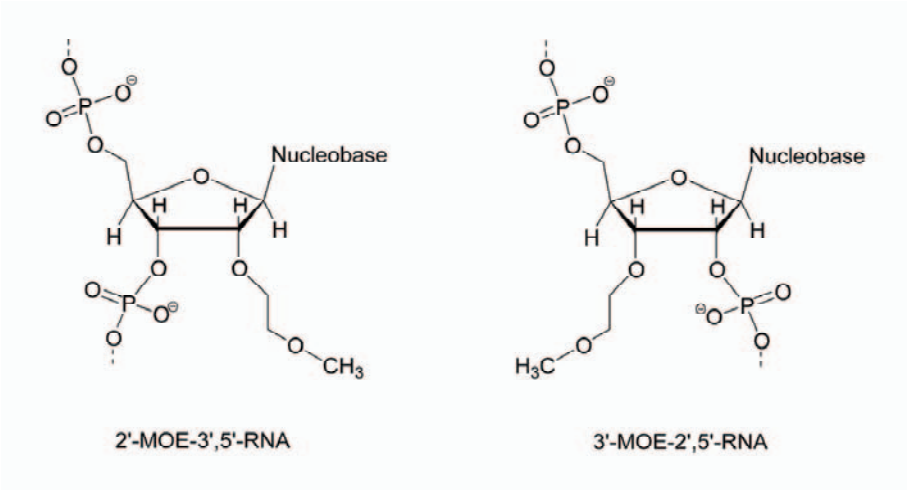
RNA and DNA molecules form complex, three-dimensional structures that participate in many biological processes, including storage and information transfer, perform catalysis and are recognized by other molecules in the cell. Modification of nucleic acids structures can enhance properties which are already present in them. In addition they can bestow new properties which natural RNA and DNA do not have. Such new properties can be useful for practical application of nucleic acids in medicine, biology, biochemistry and biotechnology. Knowledge of three-dimensional structures of nucleic acids is essential for understanding their biological functions.

The dissertation presents solution structures of two modified oligonucleotides and describes the impact of modifications in the sugar-phosphate backbone on the interstrand association of nucleic acids. The conformational preorganization of the oligonucleotide strand to binding conformation prior to complexation with other molecule is influenced by the choice of substituents on sugar-phosphate backbone and heterocyclic nucleobase. In this way, we studied the solution structures of two modified *CG*CGAA*U*U*CG*CG oligonucleotides with different phosphodiester linkages using nuclear magnetic resonance spectroscopy (NMR) and molecular dynamics simulations (MD). This sequence serves as a benchmark for both experimental and theoretical studies in structural biology of nucleic acids. Both oligonucleotides bear the same substituents on the ribofuranosyl moiety, except that methoxyethyl (MOE) is placed on 3'-position of sugar ring in first oligonucleotide, and on 2'-position in the second oligonucleotide. All cytosines and uracils are methylated at position C5.

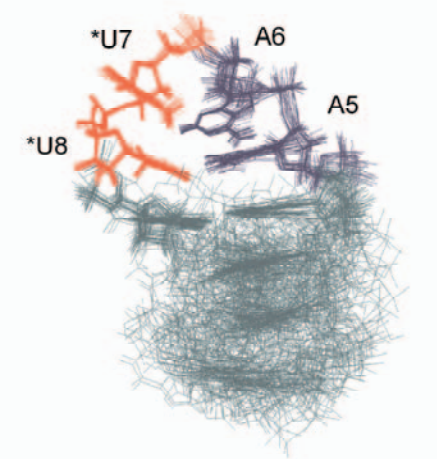


2'-MOE-3',5'-RNA 3'-MOE-2',5'-RNA

We have shown that 3'-MOE-2',5'-RNA exhibits structural equilibrium between hairpin and duplex structures, that is dependent upon oligonucleotide concentration, salt concentration, temperature and pH. Unusual effect of pH over a narrow physiological range is observed for imino proton resonances with exchange broadening observed at low pH and relatively sharp lines observed at higher pH. In addition, pH influences dynamics of opening and closing of base pairs of 2',5'-linked duplex. Exchange broadening of imino resonances could be explained by dynamic 'breathing', model where interstrand hydrogen bonds are formed and disrupted at the rate that is intermediate on the NMR timescale.

We were the first to describe (Plevnik et al. (2005) *Nucleic Acids Res*., 33, 1749-1759.) the solution structure of fully 2',5'-linked RNA hairpin, which displays a unique structural motif and well-defined loop. On the other hand, the stem region is more flexible. Our results oppose the traditional view of RNA hairpins in which the stem is considered to be highly structured while the loop is disordered. The hairpin loop is stabilized

by Watson-Crick A5•*U8 base pair and by n → π* stacking interactions of O4' lone-pair electrons and aromatic rings. Such stabilizing n → π* interactions are important in 2',5'-linked structures. In addition, syn orientation of terminal residues seems to be an intrinsic property of 2',5'-linked nucleic acids.



3'-MOE-2',5'-RNA hairpin structures

We have also described the solution structure of 2'-MOE-3',5'-RNA duplex (sequence *CG*CGAA*U*U*CG*CG[1], where G[1] is 2'-deoxyguanosine), which is analogous to 3'-MOE-2',5'-RNA oligonucleotide. The melting point of 2'-MOE-3',5'-RNA duplex is very high (T_m ≈ 95°C), which indicates the formation of a very stable duplex with strong base-pairing and stacking interactions. The duplex forms an A-type duplex structure, that was determined by NMR spectroscopy and MD. Comparison of structure with non-methylated analogue showed no significant change in structure upon C5-methylation of cytosines and uracils. Stacking patterns of bases were similar in both duplexes.


2'-MOE-3',5'-RNA duplex structures

Our studies illustrate large effect of sugar-phosphate backbone modifications on the folding of nucleic acids. Modified oligonucleotides are powerful molecular biological and biochemical research tools for regulation of gene expression and gene functions in vitro and in vivo. The knowledge of modifications and their impact on folding of nucleic acids structures leads to the design of better and more effective therapeutic drugs.

ŠTUDIJA ENCIMSKO KATALIZIRANE HIDROLIZE KARBOKSIMETIL CELULOZE V KONVENCIONALNIH IN SUPERKRITIČNIH MEDIJIH

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Delo predstavlja rezultate raziskav vpliva oblike encima na kinetiko encimsko katalizirane hidrolize karboksimetil celuloze (CMC), izvedene pri atmosferskem tlaku in v superkritičnem ogljikovem dioksidu (SC CO_2). Kot katalizator smo uporabili celulazo iz *Humicola insolens* v prašnati-nativni obliki in imobilizirano na sol-gel nosilec ter celulazo iz *Trichoderma sp.* v tekoči obliki. Reakcijo hidrolize smo izvajali v treh različnih tipih reaktorjev: v visokotlačnem mešalnem šaržnem reaktorju (pri atmosferskem tlaku in v SC CO_2), v kontinuiranem cevnem pretočnem reaktorju z nasutim slojem biokatalizatorja in v visokotlačnem kontinuiranem encimskem cevnem membranskem reaktorju (pri atmosferskem tlaku in v SC CO_2). Membranski reaktor smo razvili in skonstruirali v Laboratoriju za separacijske procese Univerze v Mariboru.

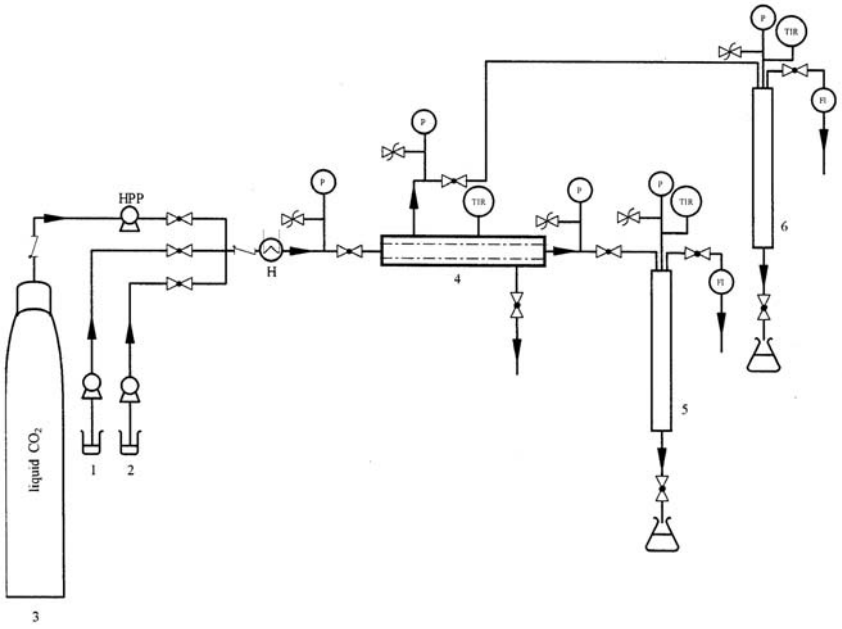
Določili smo optimalne pogoje za encimske reakcije hidrolize CMC, katalizirane z različnimi oblikami encima celulaze. Za vsako reakcijo hidrolize CMC posebej smo določili optimalno koncentracijo encima, koncentracijo substrata, temperaturo, tlak, pH, število obratov mešanja pri atmosferskem tlaku in v superkritičnem CO_2. Prav tako smo določili termodinamske parametre za posamezno reakcijo. Določili smo razpolovni čas ter stabilnost celulaze iz *Humicola insolens* (imobilizirane in surove) v superkritičnem CO_2. V cevnem pretočnem reaktorju z nasutim slojem biokatalizatorja smo proučili vpliv pretoka substrata na presnovo. Kot katalizator smo uporabili celulazo iz *Humicola insolens,* imobilizirano na aerogel.

Izvedena je bila tudi študija večkratne uporabe na aerogel imobiliziranega encima pri atmosferskem tlaku in v SC CO_2. Rezultati dokazujejo, da se encim Celluzyme 0.7T, imobiliziran na aerogel, lahko tudi do 20-krat uspešno ponovno uporabi kot katalizator za encimsko katalizirano hidrolizo CMC. Z uporabo SC CO_2 kot reakcijskega medija pa se lahko presnova celo poveča.

Na osnovi dobljenih eksperimentalnih rezultatov smo postavili matematični model encimsko katalizirane hidrolize CMC. Predpostavili smo, da je reakcija reverzibilna.
Predpostavljeni model je statistično zadovoljil naše zahteve in tako lahko rečemo, da popolnoma opisuje kinetiko encimsko katalizirane hidrolize CMC pri atmosferskem tlaku neglede na obliko in vrsto encima.

Navadno encimski procesi potekajo v šaržnih reaktorjih, pri čemer se uporabljajo encimi v njihovi topni obliki. Ti tipi reaktorjev predstavljajo visoke obratovalne stroške zaradi vsakokratnega zagona in zaustavitve procesa po končani šarži. Prav tako je potrebno izvesti naknadno separacijo produkta od nezreagiranih reaktantov in encima, kar predstavlja iz tehnološkega vidika novo operacijo v proizvodnji in s tem dodatne stroške. Rešitev predstavlja uporaba membranskega reaktorja, kjer sta reakcija in separacija združeni v eno enoto.

Material, primeren za izdelavo visokotlačnega kontiniuranega encimskega cevnega membranskega reaktorja (VKECMR), in njegove karakteristike smo določili na osnovi izračunov in predpostavljenih zahtev. Slednjega smo nato vključili v postroj za kontinuirano visokotlačno izvedbo encimsko kataliziranih reakcij (Slika 1).



Slika 1: Visokotlačni kontinuirani encimski cevni membranski reaktor (VKECMR). 1 – plin; 2, 3 – substrati; 4 – reaktor z membrano; 5, 6 – separatorja; HPP – visokotlačna črpalka; TIR – temperaturni regulator in indikator; PI – indikator tlaka; FI – indikator pretoka plina. Volumen reaktorja je 17,67 mL.

V omenjenem reaktorju, kjer smo celulazo iz Humicola insolens predhodno kovalentno vezali na keramično membrano, smo uspešno izvedli modelno reakcijo hidrolize CMC pri atmosferskem tlaku in v SC CO_2. Z uporabo SC CO_2, kot reakcijskega medija za encimsko katalizirano hidrolizo CMC v VKECMR se je presnova celo povečala.

STUDY OF ENZYME-CATALYZED HYDROLYSIS OF CARBOXYMETHYL CELLULOSE IN CONVENTIONAL AND SUPERCRITICAL MEDIA

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Influence of different forms of cellulose on the kinetics of enzyme-catalyzed hydrolysis of carboxymethyl cellulose (CMC) performed at atmospheric pressure and in the supercritical carbon dioxide (SC CO_2) is presented in this work. Cellulase from *Humicola insolens* in powder (native) form and immobilized on sol-gel carrier as well as the cellulase from *Trichoderma sp.* in liquid form were used as biocatalysts. Hydrolysis was performed in three different types of reactors: in a high-pressure batch stirred-tank reactor (at atmospheric pressure and in SC CO_2), in a continuous packed-bed reactor and in a high-pressure continuous enzyme tubular-membrane reactor (at atmospheric pressure and in SC CO_2), which was developed and constructed in the Laboratory for Separation Processes at University of Maribor.

Optimal conditions for enzymatic hydrolysis of CMC catalyzed by different forms of cellulase were determined. For each enzymatic hydrolysis of CMC the optimal concentration of enzyme, concentration of substrate, temperature, pressure, pH and rotational speed were defined at atmospheric pressure and in SC CO_2. Likewise the thermodynamic parameters for each reaction were established. The half-life and stability of cellulase from *Humicola insolens* (immobilized and native) in SC CO_2 were determined. The influence of flow of the substrates on the conversion was studied in the continuous packed-bed reactor. Cellulase from *Humicola insolens* immobilized on aerogel was used as biocatalyst.

Reuse of on aerogel immobilized enzyme was studied at atmospheric pressure and in SC CO_2. Results demonstrate that enzyme Celluzyme 0.7T immobilized on aerogel could be successfully reused up to 20-times as a catalyst for enzyme-catalyzed hydrolysis of CMC. When SC CO_2 was used as reaction medium, obtained conversion was even higher.

On the basis of obtained experimental results, mathematical model for enzyme catalyzed hydrolysis of CMC was predicted. Reversibility of the reaction was supposed. Predicted mathematical model has statistical satisfied our demands and completely describes the kinetics of enzyme catalyzed hydrolysis of CMC at atmospheric pressure apart from the shape and species of the enzyme.

Enzyme catalyzed processes are usually performed in batch reactors, where enzymes are used in their soluble form. These types of reactors present high working costs because of the starting and stopping steps after each ended batch. Likewise, the additionally separation of the products from unreacted substrates and enzyme is necessary. From industrial point of view this presents a new operation in production and consequently additional costs. The solution of this problem is presented by using the membrane reactor where reaction and separation are united in one unit.

Proper material for the construction of the high-pressure continuous enzyme tubular-membrane reactor (HPCETMR) and the characteristics of the reactor were determined on the basis of the calculations and demands. The reactor was included in the equipment for continuous high-pressure performance of the enzyme-catalyzed reactions (Figure 1).

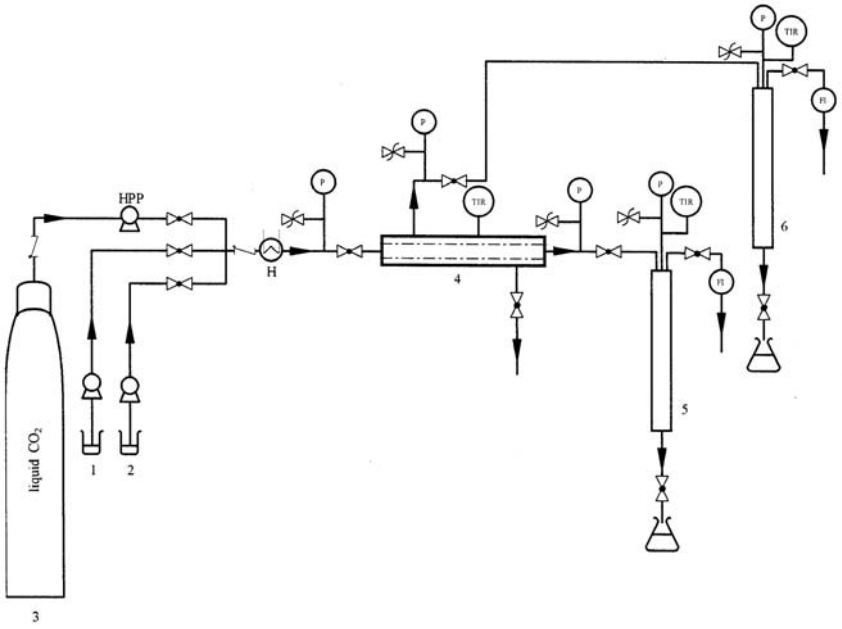

Figure 1: High-pressure continuous enzyme tubular-membrane reactor (HPCETMR). 1 – gas; 2, 3 – substrates; 4 – reactor with membrane; 5, 6 – separators; HPP – high-pressure pump; TIR – temperature regulator and indicator; PI – pressure indicator; FI – flow rate indicator. Volume of the reactor was 17.67 mL.

In the above mentioned reactor, where cellulase from *Humicola insolens* was previously covalent bound on the ceramic membrane, the model reaction of hydrolysis of CMC at atmospheric pressure and in SC CO_2 was performed. The conversion increased when SC CO_2 was used as reaction medium for enzyme-catalyzed hydrolysis of CMC in HPCETMR.

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OPREDELITEV VRSTE KVASOVK RODOV *Hanseniaspora* IN *Kloeckera* NA OSNOVI POLIFAZNE TAKSONOMIJE

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Število vrst kvasovk, za katere so dokazali, da povzročajo bolezni ljudi in živali kot oportunisti, pridobiva vse večjo pozornost. Za njihovo hitro in zanesljivo identifikacijo je potrebna predhodna postavitev klasifikacijskega sistema, ki odraža naravne odnose med vrstami. Za postavitev takega sistema smo v našem delu izbrali polifazni pristop, pri katerem smo opredeljevali vrste na podlagi njihovih genotipskih, fenotipskih in filogenetskih značilnosti. To je obsegalo primerjavo rezultatov analize nukleotidnega zaporedja ribosomskih genov in genov, ki kodirajo proteine, meritev homologije DNA-DNA, analize RAPD-PCR in AFLP, restrikcijske analize ribosomskega gena, elektroforetske kariotipizacije in fiziološke karakterizacije 106 apikulatnih sevov rodov *Hanseniaspora* in *Kloeckera*. Štirinajst sevov kvasovk se je genetsko razlikovalo od ostalih vrst rodu *Hanseniaspora* in na podlagi rezultatov merjenja hololog ije DNA-DNA smo opisali pet novih vrst rodu *Hanseniaspora*. Za rekonstrukcijo filogenije rodov *Hanseniaspora* in *Kloeckera* ter uvrstitev novih vrst v filogenetski okvir smo rekonstruirali filogenetske odnose med vrstami na podlagi nukleotidnega zaporedja dveh ribosomskih regij in dveh genov, ki kodirajo proteine. Za identifikacijo vrst smo elektroforetske profile restrikcijskih vzorcev regij ITS vseh enajstih vrst *Hanseniaspora* in *Kloeckera* predstavili v obliki stopenjske identifikacijske sheme oz. »molekularnega ključa«.

SPECIES DEFFINITION OF THE YEAST GENERA *Hanseniaspora* AND *Kloeckera* ON THE BASIS OF POLYPHASIC TAXONOMY

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Number of human and animal pathogenic yeasts has received considerable attention in recent years. The basis for accurate and rapid identification is a classification system which reflects natural relationships. In order to approach such system we used polyphasic approach which integrates genotypic, phenotypic and phylogenetic information of an organism for species definition of the genera *Hanseniaspora* and *Kloeckera*. Comparative 26S rDNA, ITS, actin and EF-1α sequence analysis, DNA-DNA hybridization experiments, RAPD-PCR and AFLP fingerprinting, electrophoretic karyotyping and physiological characterisation were performed on 106 strains of *Hanseniaspora* and *Kloeckera* species. Fourteen strains were genetically distinct from currently described species of *Hanseniaspora*. The taxonomic status of these strains was checked by using DNA ressociation analysis which showed that these strains are representatives of five novel species of Hanseniaspora. For the phylogenetic placement of the novel *Hanseniaspora* species and for phylogenetic reconstruction of the genera we tested four different phylogenetic markers, two regions of the ribosomal gene and two protein-encoding genes. As a reliable method for species identification the restriction patterns of the internal transcribed regions (ITS) were presented in a form of a molecular identification key.

MULTIPLE KSILANAZE VAMPNE BAKTERIJE *Pseudobutyrivibrio xylanivorans* Mz5^T IN MOŽNOSTI NJENE PROBIOTSKE UPORABE

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Vampna bakterija *P. xylanivorans* Mz5^T ima zelo aktiven encimski sistem za razgradnjo ksilana. Ksilanaze pogosto uporabljajo kot krmne dodatke za povečanje izkoristka krme in izboljšanje zdravstvenega stanja živali, hkrati pa so med najpomembnejšimi encimi v več biotehnoloških panogah. Zato je bil namen dela natančneje preučiti ta sistem in probiotske lastnosti seva. Na ksilanogramih smo zaznali do 14 različnih ksilanolitično aktivnih proteinov (MW 30–146 kDa). S kombinacijo različnih kromatografij smo izolirali 30 kDa ksilanazo, delno očistili pa še 44, 58 in 81 kDa encime. Najmanjša je bila katalitska domena ksilanaze XynT iz družine glikozil-hidrolaz 11, ki je prvi odkriti encim iz te družine pri butirivibrijih. Najaktivnejša je bila pri 38° C in pH 5,6, produkti hidrolize so bili večji ksilooligosaharidi. Gen *xynT* kodira protein 602 aminokislin, ki ima poleg ksilanazne še dve domeni: prva je podobna domenam za vezavo na polisaharide, druga pa polisaharid-deacetilaznim domenam. 44 kDa ksilanaza je najaktivnejša pri 38° C in pH 6, produkti hidrolize so manjši ksilooligosaharidi in ksiloza. Proučevani sev ima tudi druge probiotske lastnosti: ustvarja ugodno kislo okolje s tvorbo mlečne in maslene kisline; uspešno se pritrja na modelne epitelne celice Caco-2; tvori bakteriocinom podobne snovi, ki inhibirajo rast nekaterih potencialnih patogenih bakterij; in je udeležen v procesu biohidrogenacije linolne kisline, katere produkt je *trans*-vakcenična kislina, ki se v tkivu mlečne žleze pretvori v koristno konjugirano linolno kislino (CLA). Pridobljeni rezultati opravičujejo nadaljnja *in vivo* proučevanja probiotske uporabe *Pseudobutyrivibrio xylanivorans* Mz5^T v prehrani živali.

MULTIPLE XYLANASES OF RUMEN BACTERIUM *Pseudobutyrivibrio xylanivorans* Mz5^T AND ITS POSSIBLE USE AS A PROBIOTIC

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Rumen bacterium *P. xylanivorans* Mz5^T has a very potent enzyme system for xylan degradation. Xylanases are broadly used as feed additives to improve feed degradation and health performance of the animal. They are also among the most important industrial enzymes. Therefore the aim of the present work was to study this system and also probiotic properties of the strain Mz5^T. We were able to detect up to 14 xylanolytically active proteins on xylanograms (MW 30–146 kDa). Combination of different chromatographies allowed us to isolate 30 kDa xylanase, and partially purify 44, 58 and 81 kDa enzymes. The smallest one was a catalytic domain of xylanase XynT from family 11 of glycosyl hydrolases. XynT is the first enzyme from this family in butirivybria. It was the most active at 38° C and pH 5.6, hydrolysis products are bigger xylooligosaccharides. Gene *xynT* is coding for protein of 602 amino acids and has, in addition to xylanase domain, also two others: one domain is resembling carbohydrate binding domains, the other polysaccharide deacetylase domains. 44 kDa xylanase was the most active at 38° C and pH 6, hydrolysis products are smaller xylooligosaccharides and xylose. Studied strain has also some other probiotic properties: it creates acidic environment by butyric and lactic acid production; it successfully attaches to model epithelial cells Caco-2; it produces bacteriocin-like substances which inhibit some potential pahtogenic bacteria; and is involved in biohydrogenation of linoleic acid to trans-vaccenic acid, which is then transformed in mammary tissue to beneficial CLA. These results justify further *in vivo* tests for probiotic use of *Pseudobutyrivibrio xylanivorans* Mz5^T in animal nutrition.

TKIVNO-SPECIFIČNO URAVNAVANJE BIOSINTEZE HOLESTEROLA V MODIH, JETRIH IN MOŽGANIH V RAZLIČNIH FIZIOLOŠKIH/PATOFIZIOLOŠKIH POGOJIH

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Sodoben način življenja in z njim povezane metabolične motnje imajo v razvitem svetu že značilnosti epidemije in so najpogostejši vzrok srčno-žilnih obolenj. Vse več dejstev kaže, da je patogeneza teh bolezenskih stanj posledica prepletanja imunskega odgovora in metabolizma lipidov. Zato smo z doktorskim delom želeli ugotoviti vpliv vnetnega citokina TNFα na homeostazo holesterola v jetrih, možganih in modih miši. Ugotovili smo, da vnetni citokin TNFα poseže v mehanizem prilagajanja organizma na stradanje in aktivira anabolno pot sinteze holesterola. Pod vplivom TNF1α se pri stradanih živalih v vseh treh tkivih poveča količina sterolnih intermediatov. V jetrih s TNFα aktivirano izražanje cyp51 mRNA vodi v povečano količino tako proteina cyp51 kot tudi njegovega produkta FF-MAS. Analize transkriptoma nakazujejo, da je v jetrih aktivacija prepisovanja holesterogenih genov s TNFα neodvisna od količine holesterola, saj izražanje glavnih regulatornih genov povratne zanke holesterola ni spremenjeno. Na ravni serumskih lipidov TNFα zniža količino HDL-holesterola, kar je posledica negativnega uravnavanja izražanja apolipoproteinov, značilnih za HDL. Poveča pa se izražanje serumskih amiloidnih proteinov SAA, ki zamenjajo apoA1 v delcih HDL in s tem delce HDL spremenijo iz zaščitnih v pro-aterogene.

TISSUE-SPECIFIC REGULATION OF CHOLESTEROL BIOSYNTHESIS IN THE TESTIS, LIVER AND BRAIN IN DIFFERENT PHYSIOLOGICAL/PATHOPHYSIOLOGICAL CONDITIONS

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Contemporary lifestyle leads to different metabolic disorders that already have epidemic characteristics and often lead to cardiovascular diseases. There is increasing evidence that pathogenesis results from interactions between the immune system and lipid metabolism. Therefore, we wanted to ascertain the impact of inflammatory cytokine TNFα on cholesterol homeostasis in the mouse liver, brain and testes. Inflammatory cytokine TNFα interferes with adaptation processes to starvation and activates anabolic pathway of cholesterol synthesis. Rise of the quantity of sterol intermediates after TNFα stimuli in all three tissues of fasted animals has been detected. In the liver, TNFα-induced up-regulation of cyp51 mRNA leads to the increased protein level as well as increased amount of FF-MAS. Transcriptome analysis indicated that TNFα-induced cholesterogenic genes expression in a cholesterol-level independent manner, since the expression of main cholesterol feedback regulatory proteins is not altered. At the level of serum lipids, TNFα-induced a decrease of HDL-cholesterol as a consequence of down-regulation of HDL apolipoproteins. However, TNFα-activated the expression of serum amyloid proteins (SAA) that can replace apoA1 as the predominant apoprotein on the HDL particles and change them from protective to pro-atherogenic.

SUBTRAKCIJSKA KNJIŽNICA DIFERENCIALNO IZRAŽENIH GENOV PRI RAZVOJU RAKA ŽELODCA

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Rak želodca je izredno heterogena bolezen in čeprav je bilo do sedaj odkritih veliko genov, ki so povezani z njegovo patogenezo, je o natančnih poteh nastanka zelo malo znanega. Namen raziskave je bil odkriti spremenjene odtise različno izraženih genov v tumorskih in normalnih tkivih bolnikov z rakom želodca in pri bolnikih s kroničnim gastritisom, s pomočjo izdelave knjižnic cDNA. Za izdelavo le-teh smo uporabili metodo zaviralne subtrakcijske hibridizacije (SSH).
Pregled posameznih knjižnic cDNA diferencialno izraženih genov je pokazal razlike v izražanju genov med tumorskimi in normalnimi tkivi bolnikov z rakom želodca in prav tako so se pokazale razlike med tkivi teh bolnikov in bolnika s kroničnim gastritisom. Iz profila dobljenih spremenjenih genov lahko povzamemo, da se v tumorskih celicah okvari veliko signalnih poti in celičnih procesov, ki so potrebni za normalno homeostazo. Odkrili smo tudi spremenjeno izražanje gena CHES1, ki sodeluje pri uravnavanju celičnega cikla in ga do sedaj po podatkih iz literature še niso povezovali z rakom želodca.
Metoda SSH bi lahko bila uporabna za določanje molekularnih podpisov, značilnih za posamezne podtipe raka želodca, kar bi omogočilo izbiro ustreznega zdravljenja bolnikov, poleg tega pa bi v sodelovanju s farmacevtsko industrijo omogočala tudi razvoj novih, bolj učinkovitih kemoterapevtikov, in razvoj posameznikom prilagojenega načina zdravljenja.

SUBTRACTION LIBRARY OF DIFFERENTIALLY EXPRESSED GENES ASSOCIATED WITH THE DEVELOPMENT OF GASTRIC CANCER

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Gastric cancer is a very heterogeneous model disease and although much has been learned about the DNA changes that eventually lead to its development, the detailed mechanisms still remain unclear. The purpose of this study was to identify gene expression profiles in tissues of gastric cancer and in biopsies of gastric precancerous lesions by constructing a cDNA library with suppressive subtraction hybridization (SSH).
Data analyses of differentially expressed genes showed differences in gene expression in tumor tissues compared to corresponding normal mucosa and also differences between precancerous lesions compared to gastric tumor tissues. The profile of obtained differentially expressed genes showed the deregulation of many signaling pathways and cellular processes that are involved in homeostasis of normal gastric epithelium. Additionally, we discovered down-regulation of CHES1, a cell cycle regulator. To our knowledge this gene has not been brought into context with tumorigenesis of gastric cancer yet.
The SSH method could be used to examine molecular signatures of patients and thus help the clinicians to define subtypes of gastric cancer, predict their biological behavior and select the suitable chemotherapy. In addition, it could help to determine new molecular targets for the development of effective therapies adapted to individual patients.

STRUKTURNE ŠTUDIJE OLIGOMEROV IN AMILOIDNIH FIBRIL STEFINOV A IN B

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Molekularni in celični mehanizmi nevrodegenerativnih bolezni, kot so na primer Alzheimerjeva in Parkinsonova bolezen in nededne oblike prionskih bolezni, so verjetno skupni. Eden od pomembnih mehanizmov nevrodegeneracije je napačno zvijanje proteinov, ki posledično agregirajo in tvorijo amiloidne fibrile. Amiloidne fibrile različnih proteinov imajo enako zunanjo morfologijo in notranjo strukturo protofilamenta. Odločili smo se za študij mehanizma fibrilacije s strukturnega vidika na dveh homolognih, netoksičnih proteinih, stefinih A in B in njunih mutantih. Ugotovili smo, da dimenzije ogrodja fibril stefinov A in B in značilni difrakcijski vzorec β-struktur znotraj fibril ustrezajo splošno znanim karakteristikam amiloidnih fibril vseh proteinov, vendar stefin B veliko lažje tvori fibrile kot stefin A. Da bi pojasnili, zakaj je temu tako, smo analizirali znane tridimenzionalne strukture in modele monomerov in prepletenih dimerov. Na podlagi že znanih študij zvijanja in fibrilacije himernih in točkovnih mutantov stefinov A in B in rezultatov študij oligomernega stanja stefinov A in B in njunih mutantov, smo se odločili za kristalizacijo točkovnega mutanta stefina B P79S, pri katerem je bil prolin na mestu 79 zamenjan s serinom. Kristalna struktura je pokazala, da je ta mutant tetramer, sestavljen iz dveh prepletenih dimerov, ki ju sestavljata po dve domeni z ohranjenim cistatinskim zvitjem. Na podlagi rešene tridimenzionalne strukture točkovnega mutanta stefina B in izmerjenih dimenzij fibril ter ostalih biokemijskih študij predlagamo model zlaganja tetramerov točkovnega mutanta stefina B v fibrile. Pričakujemo, da bo natančnejše poznavanje mehanizma proteinske agregacije in posledične toksičnosti za celice vodilo do bolj racionalnega načrtovanja zdravil.

STRUCTURALS STUDIES OF OLIGOMERS AND AMYLOID FIBRILS OF STEFINS A IN B

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Common cellular and molecular mechanisms underlie different neurodegenerative diseases, from Alzheimer's disease, Parkinson's disease, to sporadic prion diseases. The molecular mechanisms include aberrant protein folding and aggregation in the form of amyloid fibrils. Under properly chosen conditions proteins in general form amyloid fibrils. Amyloid fibrils of different proteins share a common molecular skeleton, the protofilament core structure, which is a continuous β-sheet helix. Two homologous nontoxic proteins stefins A and B were chosen to study the mechanism of fibrillation from a structural point of view. Although topologically similar, they are subjected to different folding pathways. We demonstrated that both stefins share common structural features characteristic for other amyloid fibrils, but the conditions needed to undergo fibrillation differ. Analysis of three-dimensional structures of monomers and domain-swapped dimers suggest that major differences in stability of both homologues stem from arrangement of specific salt bridges, which fix α-helix to β-sheet in stefin A monomeric and dimeric forms. Data from folding and fibrillation studies of stefins A and B and their mutants and results of oligomeric state studies of stefins led us to crystallize a stefin B point mutant P79S. We solved the stefin B point mutant P79S crystal structure. The crystal structure revealed that stefin B point mutant P79S is a tetramer formed by two domain-swapped dimers. The three-dimensional structure of the tetramer and the dimensions of the stefin amyloid fibrils measured by atomic force microscopy gave us some insights into the mechanism of fibrillation. As a result of our investigation we propose that a tetramer is the next intermediate of fibrillation and that linear aggregation of tetramers takes place afterwards to form a protofibril. We believe that deeper understanding of the detailed mechanism of protein aggregation and cellular toxicity should lead to rational drug design for this type of diseases.

POVEZAVA MED STRUKTURO IN DELOVANJEM GLIVNE 17ß-HIDROKSISTEROID-DEHIDROGENAZE, MODELNEGA ENCIMA NADDRUŽINE KRATKOVERIŽNIH DEHIDROGENAZ/REDUKTAZ

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Redukcija 17-ketosteroidov je ekonomsko pomemben biokatalitični proces pri proizvodnji steroidnih zdravilnih učinkovin. To stopnjo v farmacevtski industriji katalizirajo različni mikroorganizmi in rekombinantni encimi. Oksidoredukcije androgenov in estrogenov v prisotnosti koencima NADP(H) katalizira tudi 17β-hidroksisteroid-dehidrogenaza iz glive *Cochliobolus lunatus* (17β-HSDcl), encim proteinske naddružine kratkoverižnih dehidrogenaz/reduktaz. S študijo stabilnosti encima smo našli ustrezne pogoje kristalizacije ter delno razrešili kristalno strukturo. Ugotovili smo, da se podenoti 17β-HSDcl povezujeta v aktivno dimerno obliko preko Q-osi, ki vključuje aminokisline αF- in αE-vijačnic. Z usmerjeno mutagenezo smo zamenjali Val161 ter Tyr212, ki se nahajata na področju za vezavo substrata, ter tako izboljšali začetne hitrosti reakcij za redukcijo androstendiona v testosteron. Z zamenjavo Tyr49 v Asp, ki se nahaja na področju za vezavo koencima, smo spremenili koencimsko specifičnost encima. Z zamenjavo His164, ki je del manj gibljivega pokrova, ki pokriva aktivno mesto, pa smo dobili encim s povečano katalitično aktivnostjo. Proučevali smo tudi potencialne inhibitorje encima. Med fitoestrogeni so bili najbolj učinkoviti hidroksilirani flavoni, sledijo pa kumestan kumestrol, izoflavoni ter flavanoni. Tudi strukturno podobni estri cimetne kisline inhibirajo 17β-HSDcl.

STRUCTURE/FUNCTION RELATIONSHIP IN FUNGAL 17ß-HYDROXYSTEROID DEHYDROGENASE, A MODEL ENZYME OF THE SHORT-CHAIN DEHYDROGENASE/ REDUCTASE SUPERFAMILY

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17-Ketosteroid reduction is a biocatalytical process of economic significance for the production of steroidal drugs. This reaction can be catalyzed by different microorganisms or by recombinant enzymes. Also 17β-HSD from the fungus *Cochliobolus lunatus*, member of the short-chain dehydrogenase/reductase superfamily, catalyses reversible oxidoreductions of androgens and estrogens in the presence of the coenzyme NADP(H). With stability studies we determined optimal conditions for enzyme crystallization and the partially refined crystallographic structure of the enzyme was resolved. Our results revealed that the dimerization takes place across the Q-axis interface, formed by the αE and αF helices, and is prerequisite for catalysis. Replacement of Val161 and Tyr212 in the substrate-binding region increased the initial rate for conversions of androstenedione to testosterone. Replacement of Tyr49 within the coenzyme-binding site with Asp changed the coenzyme specificity of the enzyme. The replacement of His164, situated in the nonflexible part of the lid that covers the active center, resulted in an enzyme that possessed a higher catalytic activity. We also searched for the potential inhibitors of the enzyme. Among the phytoestrogens tested, the best inhibitors were hydroxylated flavones, coumestan coumestrol, isoflavones and flavanones. Structurally related cinnamic acid esters were potent inhibitors of the fungal 17β-HSDcl.

LPS-VEZAVNI PROTEINI: ANALIZA LASTNOSTI IN INTERAKCIJ Z ENDOTOKSINOM

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Najbolj proučen stimulator človeškega imunskega sistema je bakterijski lipopolisaharid (LPS), ki lahko ob pretiranem odzivu organizma privede do septičnega šoka. V krvnem obtoku LPS vežeta proteina LBP in CD14 ter preneseta LPS do kompleksa MD-2/TLR4, ki prenese signal LPS v celico. MD-2 sam veže LPS in je nujno potreben za odziv na LPS. Identificirali smo fragment MD-2, ki je bil sposoben vezati LPS in je imel antimikrobno delovanje. Na podlagi strukture proteina Der p 2 smo postavili model 3D strukture MD-2. Pravilnost modela so potrdile lastnosti ciljanih mutant MD-2 in rekombinantnega MD-2 (bMD-2), pridobljenega iz bakterij. Mutacije aminokislin (AK) lizin in arginin na področjih, kamor naj bi se vezal LPS, so zmanjšale odziv na LPS. bMD-2 je bil aktiven, čeprav ni glikoziliran. Protein ima tudi podoben delež β-strukture kot model, vsebuje tri disulfidne mostičke in je zelo temperaturno stabilen. Strukturno je torej MD-2 podoben Der p 2, vendar za razliko od MD-2 Der p 2 le šibko veže LPS.
Na osnovi AK zaporedja HBPBH (Hidrofobna, Bazična in Polarna AK) smo identificirali še en protein, ki je vezal LPS. Efektorska domena (ED) in heptapeptidni fragment proteina MARCKS sta vezala LPS in zavirala izločanje citokina TNFα po stimulaciji z LPS. Pokazali smo neposredno *in vivo* interakcijo proteina MARCKS in LPS. LPS-vezavno mesto je znotraj ED in se prekriva z aktin-vezavnim mestom. LPS je preprečil polimerizacijo aktina z ED in povzročil depolimerizacijo F-aktina, kar kaže vlogo interakcije LPS/MARCKS v oblikovanju citoskeleta.

LPS-BINDING PROTEINS: ANALYSIS OF THEIR PROPERTIES AND INTERACTIONS WITH ENDOTOXIN

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Most known stimulator of the human immune system is bacterial lipopolysaccharide (LPS), which can lead to a condition called septic shock. In blood circle LPS is bound by extracellular proteins LBP and CD14. LPS is then transmitted to MD-2/TLR4 complex, which transmits the LPS signal into the cell. MD-2 is capable of binding LPS itself and is essential for the LPS signaling. We have identified a fragment of MD-2, capable of binding LPS and was having antimicrobial activity. We have prepared a 3D structure model of MD-2 based on the protein Der p 2. The model was confirmed using site-directed MD-2 mutants and bacterially expressed recombinant MD-2 (bMD-2). Mutations of amino acids (AAs) lysine and arginine, which are situated at the entrance of the proposed LPS-binding site, decreased MD-2 activity. bMD-2 was active in spite of the lack of glycosylation. In agreement with the model bMD-2 contains β-type secondary structure, three disulphide bridges and is temperature stable, what makes him structurally similar to Der p 2. A main difference between the proteins is a capability of binding LPS. Der p 2 weakly binds LPS.
On the basis of HBPBH AA sequence (Hydrophobic, Basic and Polar AA) another protein was identified, which bound LPS. The effector domain (ED) and the heptapeptide segment of MARCKS bound LPS and inhibited TNFα secretion after the stimulation with LPS. *In vivo* interaction between MARCKS and LPS occurred. LPS-binding site was identified inside the ED and overlaps actin-binding site. LPS inhibited polymerization of actin with ED and caused depolymerization of F-actin. This implies a new role of LPS/MARCKS interaction on cytoskeleton rearrangements.

TERMIČNA INAKTIVACIJA IN BIOKEMIČNA KARAKTERIZACIJA BIOLOŠKO AKTIVNIH SUBSTANC HIPERTERMOFILNE ARHEJE *Aeropyrum pernix*

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Hipertermofilne arheje so skupina ekstremofilnih organizmov, ki optimalno rastejo pri temperaturah nad 80 °C. V predstavljenem delu smo z uporabo različnih tehnik ugotavljali termično inaktivacijo biološko aktivnih substanc hipertermofilne arheje *Aeropyrum pernix*. Z diferenčno dinamično kalorimetrijo (DSC) smo pridobili tipičen DSC termogram celih celic *A. pernix*. Na njem so v temperaturnem območju med 100 in 150 °C vidni štirje prehodi v endotermni smeri, dva velika in dva manj izrazita. Denaturacijske spremembe, ki sovpadajo z obema velikima vrhovoma, so nepovratne in odgovorne za termično inaktivacijo organizma, kar smo preverili z zmožnostjo vzpostavitve rasti po segrevanju vzorcev do različnih temperatur. Večji del teh sprememb je povezanih z denaturacijo ribosomov. Iz talilnih krivulj izolirane DNA v pufrni raztopini (pH 7) in prisotnosti različnih koncentracij NaCl sklepamo, da je za vrh pri 102 °C na DSC termogramu celic *A. pernix* najverjetneje odgovorno taljenje DNA. S fluorescenčno spektrometrijo smo ugotovili veliko termično stabilnost liposomov, pripravljenih iz izoliranih lipidov *A. pernix*, saj prepuščanja kalceina nismo opazili niti po daljši izpostavljenosti visoki temperaturi. V koncentratih gojišča po gojenju *A. pernix* smo ugotovili tudi proteolitično aktivnost na agregate peptida P1, ki je del človeškega prionskega proteina. NaDS elektroforeza vzorca z največjo aktivnostjo je pokazala prisotnost več proteinov, možnih kandidatov za izolacijo termostabilne in termoaktivne proteinaze.

THERMAL INACTIVATION AND BIOCHEMICAL CHARACTERIZATION OF BIOLOGICALLY ACTIVE SUBSTANCES FROM HYPERTHERMOPHILIC ARCHAEON *Aeropyrum pernix*

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Hyperthermophilic archaea, a group of extremophilic organisms, grow optimally at temperatures above 80 °C. In the presented research thermal inactivation of biologically active substances from hyperthermophilic archaeon *Aeropyrum pernix* was studied by use of different techniques. Differential scanning calorimetry (DSC) was used to assess a typical DSC thermogram of *A. pernix* whole cells It shows two major and two minor overlapping endothermic transitions in the temperature range from 100 to 150 °C. Denaturation processes associated with major peaks on whole cells thermogram are irreversible and lethal for the organism, as determined with viability tests. Large part of these processes is connected to denaturation of ribosomes. Based on melting curves of isolated DNA in buffer solution (pH 7) and presence of different concentrations of NaCl we propose that the reversible transition with peak maximum at 102 °C on the thermograms of whole *A. pernix* cells coincides with melting of DNA. Flourescence spectrometry data show significant thermal stability of liposomes prepared from isolated *A. pernix* lipids, since no leaking was observed even after prolonged incubation at high temperature. Proteolytic activity against aggregates of peptide P1, which is a part of human prion protein, was determined in concentrates of growth medium after growth of *A. pernix*. There were several proteins, possible thermostable and thermoactive proteinases, present in SDS gels of the most active concentrate.

STRUKTURNO PODPRTO NAČRTOVANJE NOVIH INHIBITORJEV DNA GIRAZ Z DELOVANJEM NA ATP-VEZAVNEM MESTU

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DNA giraza je bakterijski encim, ki sodeluje pri podvajanju in prepisovanju molekul DNA. Ker je prisotna le v prokariontih, predstavlja učinkovito tarčo protimikrobnih snovi. Kvercetin je naravni flavonoid, ki zavira girazno aktivnost uvajanja dodatnih zavojev v DNA. Zato so menili, da je mehanizem inhibicije soroden kinolonom. Pokazali smo, da se kvercetin veže na 24kDa N-terminalni fragment DNA giraze B (GyrB24) in zavira ATPazno aktivnost podenote GyrB. Na osnovi sorodnosti vezavnih mest za ATP in kvercetin pri tirozin-kinazah in rezultatov NMR spektroskopije smo postavili tridimenzionalni model kompleksa GyrB24-kvercetin. V drugem delu smo za razvoj novih ATPaznih inhibitorjev uporabili metodo *de novo* fragmentnega načrtovanja učinkovin. Z merjenjem ^{15}N-HSQC spektrov izotopsko označenega proteina smo potrdili vezavo 2-aminobenzimidazola in indolin-2-ona ter določili njuni vezavni afiniteti. Z virtualnim rešetanjem podatkovne zbirke več kot 2 milijona sintetiziranih spojin smo nato iskali substituirane analoge indolin-2-ona in tako odkrili več inhibitorjev girazne aktivnosti dodatnega zvijanja DNA v mikromolarnem območju. Vezavo spojine vodnice HTS05063 v ATPazno domeno tega proteina smo dodatno potrdili z merjenjem fluorescence, masno spektrometrijo in NMR spektroskopijo. Predvideni model vezave spojine vodnice HTS05063 na osnovi eksperimentalnih študij in računalniških pristopov pojasnuje kompetitivni inhibitorni mehanizem za vezavo ATP-ja in je odlično izhodišče za nadaljnji razvoj giraznih inhibitorjev.

STRUCTURE BASED DRUG DESIGN OF NOVEL ATPASE INHIBITORS OF THE DNA GYRASE

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DNA gyrase is a bacterial enzyme which is involved in DNA replication and transcription. It is found only in prokaryotes and therefore presents an effective target for antibacterial agents. Since quercetin, one of the natural flavonoids, inhibits supercoiling activity of DNA gyrase, it has been proposed that the mechanism of inhibition is similar to quinolones. We demonstrated that quercetin binds to the N-terminal 24kDa fragment of DNA gyrase B (GyrB24) and inhibits ATPase activity of GyrB. Based on the NMR spectroscopy study and close similarity of ATP and quercetin binding sites in tyrosine kinases a three-dimensional model of GyrB24-quercetin complex was constructed. In the second part we employed a *de novo* fragment-based lead discovery to design novel ATPase inhibitors. We confirmed the binding of 2-aminobenzimidazole and indolin-2-one to the enzyme and determined their binding affinities by performing the ^{15}N-HSQC experiments of the isotopically labelled protein. In the next step we have utilized virtual screening of a chemical database (over 2 million synthesized compounds) and searched for the indolin-2-one derivatives. Using this approach we identified several inhibitors of the DNA gyrase supercoiling activity in the micromolar range. The binding of lead compound HTS05063 to the ATPase domain was corroborated by fluorescence measurements, mass spectrometry and NMR spectroscopy. The predicted binding mode explains the competitive inhibitory mechanism of HTS05063 with respect to the ATP and forms a useful basis for further development.

STRUKTURNE RAZISKAVE MIKROEMULZIJ Z METODO OZKOKOTNEGA RENTGENSKEGA SIPANJA

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Doktorska disertacija predstavlja strukturne raziskave ternarnih sistemov, sestavljenih iz vode, primarnih alkoholov od etanola do 1-dekanola ter neionskega surfaktanta dodecil-poli[etilen oksid-23] etra (Brij 35). Ti sistemi so uporabni kot možni medij za biokatalitske reakcije. Aktivnost encimov je odvisna tudi od notranje strukturiranosti takšnega medija, zato je poznavanje strukture ključno pri obravnavi korelacije med encimsko aktivnostjo ter sestavo medija. Pri raziskavah smo uporabili metodi ozkokotnega rentgenskega sipanja ter dinamičnega sipanja laserske svetlobe. Rezultate meritev sipanja smo analizirali po posplošeni metodi indirektne Fourierove transformacije. Na ta način smo določili velikost in obliko interagirajočih sipalnih delcev pri različnih sestavah ternarnih sistemov. Ugotovili smo, da so sipajoče strukturne enote okrogli miceli z dimenzijami nekaj nm, ki se, odvisno od sestave, lahko podaljšujejo v eni ali dveh dimenzijah. Vzporedno z geometrijsko strukturno okarakterizacijo posameznih raziskovanih sistemov smo posredno pokazali tudi na različne vloge, ki jih v teh sistemih odigrajo posamezni alkoholi. Etanol in propanol sta se obnašala kot sotopili in delovala kot razdiralca micelov, butanol in pentanol sta kot kosurfaktanta zviševala strukturiranost sistemov, višji alkoholi pa so se pojavljali v 'mešani' vlogi kosurfaktanta in prave oljne komponente, pri čemer sta uteži obeh vlog odvisni od izdatnosti amfifilne narave posameznega alkohola. Rezultati teh raziskav, ki so bili objavljeni v dveh znanstvenih člankih (M. Tomšič, *et.al., J. Phys. Chem B*, 108, 702, 2004; M. Tomšič, *et.al., J. Colloid Interf. Sci.*, v objavi, 2005), se zelo lepo ujemajo z dosedanjimi dognanji o encimski aktivnosti pri biokatalitskih reakcijah v mikroemulzijah.

STRUCTURAL CHARACTERIZATION OF MICROEMULSIONS BY SMALL-ANGLE X-RAY SCATTERING

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Structural properties of ternary systems composed of nonionic surfactant dodecyl-poly(ethylene oxide-23) ether (Brij 35), water and various alcohols from ethanol to 1-decanol have been investigated using small-angle X-ray scattering and dynamic light scattering techniques. Strong dependence of enzymatic activity on the composition (structuration) of these reaction media has been found previously, therefore this study offers a strong basis for exploring the structure/function relationship. The scattering data were evaluated using the well-established generalized indirect Fourier transformation method. In this way, the size and the shape of interacting scattering particles could be deduced. In parallel these results (M. Tomšič, *et al., J. Phys. Chem B*, 108, 702, 2004; M. Tomšič, *et al., J. Colloid Interf. Sci.*, *in press*, 2005) also indicate various roles of simple alcohols in these systems. In this respect ethanol and propanol were confirmed to behave as micelle breaking agents, butanol and pentanol as cosurfactants elongating the scattering particles into prolate and oblate shapes, respectively, whereas even higher alcohols behaved as cosurfactants and/or real oil-components, as the later being able to enter the areas in the close vicinity of the micellar core centres with the extent dependent on their increasing hydrophobic nature.

MOLEKULARNA EPIDEMIOLOGIJA BOVINE VIRUSNE DIAREJE (BVD) V SLOVENSKIH PLEMENSKIH REJAH GOVEDI

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V študijo molekularne epidemiologije virusa bovine virusne diareje smo zajeli viruse, ki so bili razširjeni med slovenskimi plemenskimi rejami govedi v letih od 1998 do 2003. Virusno RNA smo izolirali iz inficiranih celic, izvedli reverzno transkripcijo in pomnoževanje v eni stopnji (access RT-PCR) z začetnimi nukleotidi, ki pomnožujejo 5'nekodirajočo regijo (5'NCR) in regijo N^{pro} virusnega genoma. Produkte RT-PCR smo sekvenirali v obeh regijah. Naredili smo primerjavo 128 sekvenc 5'NCR v dolžini 238–243 nukleotidov in poravnavo 80 sekvenc izolatov v regiji N^{pro} v dolžini 392 nukleotidov. S filogenetsko primerjavo izolatov v obeh regijah virusnega genoma smo dobili razdelitev virusov v štiri podtipe znotraj genotipa BVD 1. Večina virusov spada v podtipa 1f (59 izolatov / 46,2 %) in 1d (52 izolatov / 40,6 %), manj pogosti so virusi podtipov 1b (14 izolatov / 10,9 %) in 1g (3 izolati / 2,3 %). Najbolj so razširjeni virusi podtipa 1f, ki smo jih našli le v gorenjski in primorski regiji. Izolate podtipa 1d najdemo najpogosteje na Gorenjskem in Štajerskem, enega pa smo ugotovili tudi v eni reji na Primorskem, ki je bila istočasno okužena z virusom podtipa 1f. Za boljše razlikovanje zelo sorodnih izolatov, katerih sekvence so enake v 5'NCR, je primernejša regija N^{pro}, ki je bolj variabilna in zajema daljši odsek genoma ter daje višjo statistično podporo za filogenetsko razlikovanje virusov. Ta odsek virusnega genoma smo uporabili kot genetski marker za spremljanje prenosa virusov BVD med različnimi rejami.

MOLECULAR EPIDEMIOLOGY OF BOVINE VIRAL DIARRHOEA VIRUS IN SLOVENIAN BREEDING CATTLE HERDS

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Molecular epidemiology of bovine viral diarrhoea virus (BVDV) isolates, circulating between 1998 and 2003 in the breeding cattle herds in Slovenia was studied. Viral RNA was extracted from infected cell cultures, reverse transcribed and amplified by one-step access RT-PCR with primers targeting the 5'-non coding region (5'NCR) and N^{pro} region of viral genome, followed by direct sequencing of purified PCR products obtained for both genomic regions. Sequences of 5'NCR (238–243 nt) were assembled for all 128 BVDV strains, whereas the sequences of N^{pro} region (392 nt) were determined for 80 BVDV isolates. Phylogenetic analysis of each data-set revealed identical grouping of viruses into four genetic subgroups within BVDV 1. Most viruses were found in two subgroups 1f (59 isolates / 46,2 %) and 1d (52 isolates / 40,6 %), while viruses of subgroups 1b (14 isolates / 10,9 %) and 1g (3 isolates / 2,3 %) were less common. Subgroup 1f, the most common BVDV genetic group, was only found in the two Western and North-Western regions Gorenjska and Primorska. The subgroup 1d isolates were found mainly in the Gorenjska and Štajerska region and in one herd from Primorska region which was concomitantly infected with 1f virus. For closely related viruses, identical in 5'NCR, the more variable and larger data sets of N^{pro} gene sequences provided better genetic resolution, and gave also higher statistical support for phylogenetic classification of the viruses and we used them as genetic marker for tracing BVDVs from different herds.

NOVI DERIVATI CIMETNE KISLINE KOT INHIBITORJI 17ß-HIDROKSISTEROID-DEHIDROGENAZ

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17β-hidroksisteroid-dehidrogenaze (17β-HSD) katalizirajo zadnjo stopnjo biosinteze spolnih hormonov. Ker so lahko vpletene v razvoj številnih hormonsko odvisnih bolezni, predstavljajo zanimivo tarčno mesto za kontrolo koncentracije estrogenov in androgenov v organizmu. Na osnovi estrov cimetne kisline, ki so se v preteklih raziskavah izkazali kot dobri inhibitorji encima 17β-HSD iz glive *Cochliobolus lunatus* (17β-HSDcl), smo v okviru raziskovalne naloge sintetizirali serijo novih spojin in ugotavljali njihovo delovanje. Inhibitorno aktivnost sintetiziranih spojin smo testirali na rekombinantni 17β-HSDcl, modelnem encimu proteinske naddružine kratkoverižnih dehidrogenaz/reduktaz. Rezultati meritev so pokazali, da je za inhibitorno delovanje sintetiziranih spojin nujno potrebna esterska oz. amidna skupina, pri čemer so estri cimetne kisline boljši inhibitorji modelne 17β-HSD kot amidi, ter da imajo derivati 3,4,5-trimetoksicimetne kisline slabšo inhibitorno aktivnost kot derivati nesubstituirane cimetne kisline. Z optimizacijo sintetiziranih spojin bi lahko v prihodnosti razvili bolj učinkovite inhibitorje človeških 17β-hidroksisteroid-dehidrogenaz, ki bi jih uporabljali za preprečevanje in zdravljenje različnih hormonsko odvisnih bolezni, kot so nekatere oblike raka, osteoporoza, Alzheimerjeva bolezen in druga patološka stanja.

NEW CINNAMIC ACID DERIVATES AS INHIBITORS OF 17ß-HYDROXYSTEROID DEHIDROGENASES

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17β-hydroxysteroid dehydrogenases (17β-HSDs) catalyze the last step of the biosynthesis of sex hormones. Being involved in the development of many hormone-sensitive diseases, the 17β-HSDs represent an interesting target for controlling the concentration of estrogens and androgens in the organism. Based on previous research, that demonstrated that some cinnamic acid esters are potent inhibitors of 17β-HSD from the fungus *Cochliobolus lunatus* (17β-HSDcl), a series of structurally related cinnamic acid derivatives was synthesized and evaluated. Compounds were screened for the inhibition of recombinant 17β-HSDcl, a model enzyme of the short-chain dehydrogenase/reductase superfamily. We found that for good inhibitory activity ester or amide group is essential. Cinnamic acid esters were more potent inhibitors of 17β-HSDcl than cinnamic acid amides. We also proved that derivates of 3,4,5-trimethoxycinnamic acid were less potent inhibitors than derivates of unsubstituted cinnamic acid. These compounds represent promising starting points for the development of new and more efficient inhibitors of human 17β-HSDs with the potential use in prevention and treatment of hormone dependent forms of cancers, osteoporosis, Alzheimer disease and other diseases.

INHIBITORJI REKOMBINANTNIH HIDROKSISTEROID-DEHIDROGENAZ AKR1C1 IN AKR1C3

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Hidroksisteroid-dehidrogenazi AKR1C1 in AKR1C3 spadata v proteinsko naddružino aldo-keto reduktaz in sodelujeta pri biosintezi in inaktivaciji steroidnih hormonov ter sta tako povezani z nastankom hormonsko odvisnih oblik rakavih obolenj. V okviru raziskovalne naloge smo iskali inhibitorje teh dveh encimov z rešetanjem strukturno različnih spojin: različno substituiranih cimetnih kislin in njihovih derivatov (estrov, amidov), kumarinov in derivatov naftalena. Izolacijo rekombinantnih encimov smo izvedli s pomočjo bakterije *Escherichia coli* z vstavljenim plazmidom pGEX-AKR1C1 oziroma pGEX-AKR1C3, ki nosita genski zapis za fuzijski protein glutation-S-transferaza-AKR1C1 oziroma glutation-S-transferaza-AKR1C3. Inhibicijo redukcije splošnega substrata aldo-keto reduktaz 9,10-fenantrenkinona, ob prisotnosti koencima NADPH smo spremljali spektrofotometrično. Za vse spojine smo določili odstotek inhibicije, za tiste z več kot 50 odstotno inhibicijo pa tudi IC_{50} vrednost. Ugotovili smo, da so derivati naftalena dobre spojine vodnice za iskanje novih selektivnih inhibitorjev AKR1C3, estri cimetne kisline pa dobre spojine vodnice za razvoj novih selektivnih inhibitorjev AKR1C1.

INHIBITORS OF RECOMBINANT HUMAN HYDROXYSTEROID DEHYDROGENASES AKR1C1 AND AKR1C3

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Hydroxysteroid dehydrogenases AKR1C1 and AKR1C3 that belong to the aldo-keto reductase superfamily are involved in the biosynthesis and inactivation of steroid hormones and therefore play an important role in the development of hormone dependent forms of cancers. The aim of the presented work was to identify possible inhibitors of AKR1C1 and AKR1C3. We have screened structurally very different compounds: substituted cinnamic acids and their derivatives (esters, amides), coumarines and some derivatives of naphthalene. Recombinant human hydroxysteroid dehydrogenases AKR1C1 and AKR1C3 were isolated from the bacteria *Escherichia coli* transformed with plasmid pGEX-AKR1C1 or pGEX-AKR1C3 coding for fusion protein glutation-S-transferase-AKR1C1 or glutation-S-transferase-AKR1C3. The inhibitory potency of selected compounds toward recombinant AKR1C1 and AKR1C3 was measured spectrophotometrically following the reduction of a common AKR substrate 9,10-phenanthrenequinone in the presence of NADPH as a coenzyme. IC_{50} values were determined for compounds that showed more than 50% of inhibition. Naphthalene derivatives were good and selective inhibitors of AKR1C3, while cinnamic acid esters proved to be better inhibitors of AKR1C1. Both naphthalene derivatives and cinnamic acid esters are promising lead compounds for the development of selective AKR1C1 and AKR1C3 inhibitors.

TRŽNA RAZISKAVA IN OGLAŠEVALSKA STRATEGIJA ZA MOŠKO KREMO VITASKIN MAN

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Projekt o hidronegovalni kremi Vitaskin Man vsebuje izsledke raziskave in zasnovo oglaševalske kampanje o tem, kako povečati prepoznavnost, prodajo ter uporabo moške hidronegovalne kreme za obraz – Vitaskin Man.
Jeseni 2004 smo začele s tržno raziskavo; novembra izvedle fokusno skupino, kasneje pa še anketiranje. Izsledki fokusne skupine so pokazali obstoj dveh širših skupin potrošnikov. Primarno ciljno skupino predstavljajo moški, sekundarno pa ženske, ki jim kupujejo kreme. Tržna raziskava je določila štiri segmente moških, od katerih nam dva predstavljata primarni ciljni skupini, in sicer funkcionalne uporabnike ter metroseksualce. Ker obe skupini zaznamuje nekonzervativnost glede uporabe kozmetike, smo se odločile za enotna sporočila. Slogan *Vitaskin Man. Ker voda in milo nista dovolj*, je rdeča nit kampanje. Sporoča, da k osnovni negi moškega spada tudi krema za obraz. Kakovost kreme povezuje predvsem z dejstvom, da ta reši nek problem, ki ga imajo s kožo, hkrati pa jih ne sme feminizirati. Oglaševalsko akcijo bi izvedle na štiri dopolnjujoče se načine: teaserji, oglaševanje na televiziji in v tisku ter oglaševanje z oglasnimi vizitkami. Sekundarno javnost bi nagovarjale tudi prek oglasnih vizitk, v embalažah Krkinih kozmetičnih izdelkov za ženske.

MARKET RESEARCH AND ADVERTISING STRATEGY FOR MALE FACIAL CREAM VITASKIN MAN

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Vitaskin Man project contains research results and advertising campaign plan about how to enhance recognition, sale and usage of male hydrocare facial cream – Vitaskin Man.
In autumn 2004 we started market research; focus group was carried out in November and later opinion poll. The focus group outcome showed the existence of two big customer groups. Primary target group consists of men whereas in the second group there are women who buy creams for them. Market research determined four men segments, where only two represent primary target groups – these are functional users and metrosexuals. As a result of nonkonservatism of both groups we decided for a single message: *Vitaskin Man. Because water and soap aren't enough*, is a slogan which represents the gist of the campaign. Its message conveys that facial cream is a part of basic daily care. Men must be told about the functional characteristics of the cream because they buy it for a particular reason. It is important to clearly distinguish and differentiate male usage from female. Advertising campaign would be carried out in four correlated ways: teasers, TV advertising, print advertising and advertising cards. A secondary target group would be appealed by advertising cards in Krka woman's cosmetics packagings.

AVTOMATSKO RAZVRŠČANJE SLIK CELIČNIH KULTUR

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Namen in cilj raziskovalnega dela je bil raziskati metode za avtomatsko prepoznavanje in razvrščanje slik celičnih kultur na osnovi kriterija konfluentnosti in morfoloških značilnosti celic. Metode smo razvili in preizkusili na slikah endotelijskih in fibroblastnih celičnih kultur. Najprej smo želeli ločiti konfluentne kulture od sub-konfluentnih. Prepoznavanje konfluentnosti smo dosegli z metodami iskanja robov na slikah, s primerjavo statističnih momentov in Haralickovimi teksturnimi koeficienti. Metoda Haralickovih teksturnih koeficientov se je izkazala za najuspešnejšo pri razvrščanju slik po kriteriju konfluentnosti. Potem smo želeli ločiti konfluentne endotelijske celične kulture od konfluentnih fibroblastnih kultur. To smo dosegli s primerjavo Fourierovih koeficientov, z Gaborjevimi filtri in s programsko opremo Cellenger® (Definiens), s katero smo analizirali segmentirane slikovne objekte. Slednja metoda se je izkazala kot najučinkovitejša pri reševanju zastavljenega cilja. Uspešnost metod smo med seboj primerjali s Fisherjevim diskriminantnim kriterijem.

AUTOMATIC CLASSIFICATION OF CELL CULTURE IMAGES

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In the scientific and research work it is aimed to propose methods for the automated classification of cell cultures images. The image classification of endothelial and fibroblast cell cultures is composed of two main steps. First, the cell culture is classified by its confluence character. Classification is performed through edge detection, statistical moments and Haralick texture coefficients. The method based on the Haralick texture coefficients delivered results of the highest classification rate. Secondly confluent endothelial cell cultures and fibroblast cell cultures are identified. Identification is performed through Fourier descriptors, Gabor filtering and image processing software Cellenger®(Definiens), which enabled the analysis of segmented image objects. The Cellenger®delivered the highest separability value evaluated with the Fisher discriminant criterion.

VPLIV POLIMORFIZMOV V GENU ZA ESTROGENSKI RECEPTOR ALFA NA KONCENTRACIJE LIPIDOV V SERUMU PRI ZDRAVLJENJU Z RALOKSIFENOM

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Raloksifen je nehormonska učinkovina, ki spada v skupino selektivnih modulatorjev estrogenskih receptorjev. Na estrogenski receptor (ER) se veže z veliko afiniteto. Njegovo agonistično delovanje v kosteh se izkorišča pri zdravljenju osteoporoze, nekatere študije pa kažejo, da ima ugoden učinek tudi na nivo serumskih lipidov. Zanimalo nas je ali polimorfizmi v genu za ER alfa (PvuII, XbaI in mutacija P325P) vplivajo na nivo serumskih lipidov po šestmesečnem zdravljenju z raloksifenom. V klinično raziskavo je bilo vključenih 49 pomenopavznih žensk z osteoporozo, ki so se šest mesecev zdravile z raloksifenom. Koncentracije lipidov so bile izmerjene z validiranimi rutinskimi metodami pred in po zdravljenju. Genske polimorfizme smo analizirali z analizo dolžin restrikcijskih fragmentov (PvuII in XbaI) in analizo konformacijskih polimorfizmov enoverižnih DNA (P325P). Povprečne koncentracije serumskih lipidov pred zdravljenjem so bile: celotni holesterol = 6,20 mmol/L, HDL = 1,64 mmol/L, LDL = 3,89 mmol/L in trigliceridi = 1,57 mmol/L. V celotni skupini preiskovank je zdravljenje z raloksifenom znižalo koncentracije celotnega holesterola za 4% ($p=0,01$) in LDL holesterola za 8% ($p<0,001$), ni pa imelo značilnega vpliva na koncentracije HDL holesterola in trigliceridov. Ugotovili smo, da genotipi sami ne vplivajo na koncentracije serumskih lipidov niti pred niti po zdravljenju. Tako lahko zaključimo, da genske spremembe v genu za ER alfa ne vplivajo na z raloksifenom povzročeno znižanje lipidov v serumu.

THE INFLUENCE OF ESTROGEN RECEPTOR ALPHA GENE POLYMORPHISMS ON SERUM LIPID CONCENTRATIONS AT RALOXIFENE THERAPY

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Raloxifene is one of compounds called selective estrogen receptor modulators. It has a high affinity to estrogen receptor (ER). Its agonistic effect in bone is used for treatment of osteoporosis, but some studies have also shown its beneficial effect on serum lipid levels. The purpose of our study was to find out whether polymorphisms in the ER alpha gene (PvuII, XbaI and mutation P325P) affect serum lipid levels after six months treatment with raloxifene. We studied 49 postmenopausal women who had been treated with raloxifene for six months. Lipid concentrations were measured with validated routine methods. Polymorphisms were analyzed with restriction fragment length polymorphism analysis (PvuII and XbaI) and single stranded conformation polymorphism analysis (P325P). In the whole group of patients, raloxifene lowered concentration of total cholesterol for 4% ($p=0,01$) and LDL cholesterol for 8% ($p<0,001$) but had no effect on HDL cholesterol and triglycerides. Our results suggest that serum lipid levels, before and after the therapy, do not depend on genotypes. We concluded that genetic changes in the ER alpha gene do not influence the effect of raloxifene on serum lipid concentrations.

BIOLOŠKO ČIŠČENJE FARMACEVTSKE ODPADNE VODE NA PILOTNI ČISTILNI NAPRAVI

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V diplomskem delu sem preverila možnosti aerobnega biološkega čiščenja farmacevtske odpadne vode iz fermentacije antibiotika na pilotni čistilni napravi. Pred biološkim čiščenjem sem odpadno vodo ovrednotila z osnovnimi kemijskimi analizami. Ker na sposobnost biološkega čiščenja vplivata tudi strupenost in biorazgradljivost odpadne vode, sem pred poskusi na pilotni čistilni napravi določila tudi ta dva parametra. S primerjanjem vrednosti celotnega organskega ogljika, kemijske potrebe po kisiku, biokemijske potrebe po kisiku in ostalih analiz vtokov in iztokov pilotne čistilne naprave sem ugotovila učinek čiščenja in s spremljanjem obratovalnih parametrov delovanje pilotne čistilne naprave.
Rezultati preiskav odpadne vode iz proizvodnje farmacevtskih učinkovin so pokazali, da je odpadna voda ''lahko razgradljiva'' in ni nevarna za mikroorganizme aktivnega blata, ki se nanjo hitro adaptirajo. S simulacijo čiščenja farmacevtske odpadne vode na pilotni čistilni napravi sem ugotovila, da je biološko čiščenje ustrezno le pri nižjih obremenitvah sistema. Pri simulaciji A sem dosegla še zadovoljivo čiščenje pri obremenitvi 6 vol. % farmacevtske odpadne vode v mešanici s komunalno odpadno vodo, pri simulaciji B pa sem dosegla še zadovoljivo čiščenje pri obremenitvi 4 vol. %. Med procesom čiščenja je pri simulaciji A potekala tudi nitrifikacija do obremenitve 6 vol. % farmacevtske odpadne vode. Pri simulaciji B lahko na podlagi zmanjšanja vrednosti anorganskega ogljika sklepam, da je potekala nitrifikacija do obremenitve 4 vol. %. Boljše biološko čiščenje bi verjetno dosegli z uvedbo predhodnega kemijskega čiščenja.

PILOT BIOLOGICAL TREATMENT OF PHARMACEUTICAL WASTEWATER

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The aim of our study was to characterize biotreatability potential of wastewater resulting from fermentation of antibiotics. Experiments were conducted in pilot aerobic biological wastewater treatment plant. To avoid non-reliable results due to the variable quality of the investigated wastewater because of the batch production, three different wastewaters have been investigated. Prior to wastewater treatment experiments biotreatability characterization was started by general physico-chemical analysis, ready biodegradability testing and toxicity assessment. Efficiency of wastewater treatment was monitored by determination of chemical oxygen demand, biochemical oxygen demand, dissolved organic carbon in the influent and effluent of the pilot plant. The performance of the treatment plant was also checked by following operational parameters.
Investigated wastewater was readily biodegradable and non-toxic to mixed culture of activated sludge microorganisms, which were also able to adapt to incoming wastewater quickly. In first simulation experiment – Simulation A, treatment was successful up to 6 vol. % of the wastewater (added to municipal wastewater), while at second simulation experiment - Simulation B, the successful removal of organics was accomplished up to 4 vol. % of the wastewater. At higher loads of the wastewater system was overloaded and it collapsed. In both simulations also inhibition of nitrification was observed at higher loads of the wastewater. We have concluded that biological treatment could probably be improved by effective chemical pretreatment of investigated wastewater.

SINTEZA MIMETIKOV TRIPEPTIDNEGA ZAPOREDJA ARGINIL-GLICIL-ASPARAGINSKA KISLINA

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Različna patološka stanja, kot so kardiovaskularna in vnetna obolenja, metastaziranje in osteoporoza, so posledica delovanja več vrst integrinskih receptorjev, ki prepoznavajo enako aminokislinsko zaporedje RGD (arginil-glicil-asparaginska kislina) mnogih fizioloških ligandov. Tovrstno zaporedje nam je služilo kot osnovni motiv pri načrtovanju novih potencialnih antagonistov na integrinskih receptorjih. Kot distančnik smo izbrali biciklični benzoksazinski obroč, na katerega smo na mesto 6 vezali mimetik arginina, mimetik aspartata pa smo pripeli na mesto 2 preko vmesnega distančnika oz. 1,2,4-oksadiazolskega obroča. Sintetizirali smo končno spojino, ki smo jo poskušali pripraviti po dveh sinteznih pristopih. Po prvi poti smo na benzoksazinski skelet na mesto 2 pripeli najprej 1,2,4-oksadiazolski obroč in šele potem poskušali pripraviti benzamidinski del spojine na mestu 6. Tovrstna pot žal ni bila ustrezna, saj se je večstopenjska sinteza končala pri redukciji nitro skupine spojine. Spojino smo uspeli sintetizirati po drugem sinteznem pristopu. V tem primeru smo na benzoksazinski skelet najprej pripeli p-cianobenzoil klorid na mesto 6 in šele potem vezali oksadiazolski fragment. Pripravili smo različne 3,5-disubstituirane 1,2,4-oksadiazole in derivat 1,2,4-oksadiazol-5-ona kot zaščitno skupino ali obliko predzdravila za amidinsko funkcionalno skupino. Sintetizirani sintoni bodo služili za pripravo novih mimetikov RGD-peptida, končne pripravljene spojine pa bodo prispevale k poznavanju topologije vezavnega mesta.

SYNTHESIS OF ARGINYL-GLYCYL-ASPARTIC ACID TRIPEPTIDE MIMETICS

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Different pathological states like cardiovascular and inflammatory diseases, methastazing and osteoporosis are the consequence of the action of several integrin receptors that recognize the same RGD sequence on many physiological ligands. This sequence has served us as a basic motif for the design of new antagonists of integrin receptors. We choose bicyclic benzoxazine ring as a spacer, we added mimetics of arginine and mimetic of aspartate on it on sites 6 and 2. Aspartate mimetic was additionally separated from benzoxazine ring with a 1,2,4-oxadiazole ring spacer. We managed to make the final compound, which we tried to synthesize by two approaches. First we added 1,2,4-oxadiazole ring on site 2 of benzoxazine skeleton; after that we tried to prepare benzamidine part of the compound on site 6. This type of synthesis was not appropriate since the multistep synthesis ended when we tried to reduce nitro group of intermediate compound. We managed to synthesize compound by the second approach. First we added p-cianobenzoyl chloride on site 6 of benzoxazine skeleton, and after that we added oxadiazole fragment. We prepared different 3,5-disubstituted 1,2,4-oxadiazoles and compound 1,2,4-oxadiazol-5-one as a protective group or prodrug compound for amidine functional group. Synthesized compounds will serve as a base for the new RGD peptide mimetics and they will also help to understand the topology of the binding site.

ŠTUDIJ SPROŠČANJA UČINKOVINE IZ GASTROREZISTENTNIH PELET

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V generični farmacevtski industriji je preizkus raztapljanja učinkovin pomemben *in vitro* postopek za ocenjevanje in napovedovanje lastnosti določene farmacevtske formulacije. V diplomskem delu sem razvila HPLC metodi za določevanje raztopljenega omeprazola in njegovih razpadnih produktov ter ugotavljanja vplivov različnih hidrodinamskih faktorjev na hitrost sproščanja omeprazola v medijih, ki simulirajo *in vivo* pogoje. Z 0,1 M HCl sem simulirala želodčni, s pufrsko mešanico s pH 6,8 in dodatkom natrijevega dodecilsulfata (SDS) pa črevesni sok. Pri HPLC določanju sproščenega omeprazola (HPLC- metoda 1) sem dosegla selektivno ločbo (t_r omeprazola = 2,5 min), analiza je bila hitra (3,8 min). Linearnost metode je bila v koncentracijskem območju od 0,0022 do 0,0277 mg omeprazola/mL dobra, korelacijski koeficient R^2 je znašal 0,9995. Relativna napaka analiznih rezultatov je bila manjša od ± 2,0 %. S HPLC metodo-2 sem dosegla zadovoljivo ločljivost med omeprazolom in omeprazol sulfonom (ločljivost R= 3,4), metoda je bila selektivna tudi za ostale razpadne produkte omeprazola. Omeprazol se razgrajuje na neznano spojino ter na omeprazol sulfon in ufiprazol, ki sta bila identificirana s pomočjo LC-MS/MS analize. UV- spektrofotometrično določanje sproščenega omeprazola, kjer sem absorbance vzorcev merila pri absorpcijskem maksimumu λ= 306 nm, je manj primerno zaradi neselektivnosti za razpadne produkte omeprazola. Ročni oz. avtomatizirani način vzorčenja ne vpliva na hitrost sproščanja in razpada omeprazola, vpliva pa na količino nekaterih oksidacijskih razgradnih produktov.

STUDY OF DRUG RELEASE FROM GASTRORESISTANT PELLETS

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Drug dissolution from a pharmaceutical dosage form has become important *in vitro* method for evaluation and control of quality of product in generic pharmaceutical industry. Two reversed-phase high-performance liquid chromatography methods were developed to determine released omeprazole and its degradation products from gastroresistant pellets in dissolution media, simulating gastric (0,1 M HCl) and intestinal (puffer pH 6,8 with Sodium dodecyl sulfate) juices. Good selectivity and quick analysis of released omeprazole were achieved with HPLC-method 1. Linearity was obtained between concentration range 0,0022-0,0277 mg omeprazole/ mL, correlation coefficient R^2 was 0,9995. Relative error was lower than ± 2,0 %. With HPLC-method 2, a good selectivity between omeprazole and omeprazole sulphone (resolution R= 3,4) and also for other degradation products was achieved. Omeprazole degrades to unknown compound and also to omeprazol sulphone and ufiprazole, these two were identified with LC-MS/MS analysis. Spectrophotometric analysis (absorbance measured at λ= 306 nm) was found an inappropriate method for determination of released omeprazole because of interference of its degradation products. Manual or automated sampling has no influence on release and degradation of omeprazole, but does have influence on amount of some oxidative degradation products of omeprazole.

UVAJANJE METOD ZA PREISKOVANJE TRANSKRIPTOMA IN PROTEOMA REGENERACIJE OKONČIN MEHIŠKEGA AKSOLOTLA *Ambystoma mexicanum*

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Regeneracija je proces nadomestitve manjkajočega tkiva, pri katerem gre za reaktivacijo procesa razvoja. Pri odraslih vretenčarjih je sposobnost regeneracije okončine ohranjena le pri skupini repatih dvoživk - pupkih in močeradih, med katere uvrščamo tudi mehiškega aksolotla *Ambystoma mexicanum*, modelni organizem za preučevanje regeneracije. Proces regeneracije delimo na ključne tri faze: 1. faza - celjenje rane, 2. faza - dediferenciacija in tvorba blasteme in 3. faza - ponoven razvoj okončine z rediferenciacijo. V naši raziskavi smo s pomočjo modernih metod funkcijske genomike in proteomike preučevali izražanje genov pri regeneraciji okončine. Z amputacijo zadnje okončine aksolotla in nadaljnjimi reamputacijami smo pridobili tkivne vzorce iz različnih regeneracijskih faz. Transkriptom smo preučevali z metodo zaviralne subtrakcijske hibridizacije, pripravili smo knjižnice cDNA bolj izraženih genov v fazi rediferenciacije, klonom določili nukleotidna zaporedja in jih s pomočjo bioinformacijskih orodij identificirali. Od 233 bolj izraženih transkriptov smo jih 140 uspešno sekvencirali. Zaporedja smo s programom Blast prilegali z že znanimi zaporedji v genski podatkovni bazi: 18 zaporedij nima anotacije, 94 je mitohondrijskih genov, za 28 genov pa smo našli homologna zaporedja. Izvedli smo tudi začetne preiskave proteoma. Proteinske vzorce smo ločevali z 2-D elektroforezo in jih detektirali z barvanjem s srebrom.

INTRODUCTION TO METHODS FOR TRANSCRIPTOME AND PROTEOM SCREENING OF REGENERATING AXOLOTL (*Ambystoma mexicanum*) LIMB

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Regeneration is a process where a missing part of a tissue is replaced by the process of development reactivation. Among vertebrates the ability to regenerate lost limbs is unique to the urodele amphibians – newts and salamanders. *Ambystoma mexicanum* is a model organism for regeneration studies that is also classified in this group. Process of regeneration can be roughly divided into three main phases: phase 1 wound healing, phase 2 – dedifferentiation and blastema formation, phase 3 – re-development of the limb by redifferentiation. In our research we studied the process of limb regeneration by modern approaches of functional genomics and proteomics. We obtained the samples of axolotl limbs from different regeneration stages of amputation and further reamputation of the back limb of the animal. Transcriptom was studied with Suppressive Subtraction Hybridization method, cDNA library of over expressed genes in the redifferentiaition phase was prepared, obtained clones were sequenced and identified with bioinformatics tools. From 233 clones obtained, 140 were successfully sequenced. These sequences were aligned to the other known sequences from the gene bank using Blast: 18 could not be annotated, 94 were mitochondrial genes and 28 homological sequences were found. The preliminary analyses of the proteome were also done. Protein samples were separated on the 2-D electrophoreses and detected by silver staining.

MERJENJE ANTAGONISTOV SIGNALIZACIJE LPS PREKO RECEPTORSKEGA KOMPLEKSA MD-2/TLR4

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Lipopolisaharid (LPS) je poglavitna komponenta zunanje membrane po Gramu negativnih bakterij in sproži močan odziv prirojenega imunskega sistema, ki se lahko stopnjuje do septičnega šoka in smrti. Celični odziv na LPS posreduje receptorski kompleks sestavljen iz transmembranskega Tollu-podobnega receptorja 4 (TLR4) in MD-2, ki je pritrjen na zunajcelično domeno TLR4. MD-2 je sekretorni protein in neposredno veže LPS. Inhibicija LPS odvisne aktivacije celic s specifičnimi antagonisti bi lahko bila učinkovita metoda zdravljenja septičnega šoka zaradi po Gramu negativnimi bakterijami. Naša prva naloga je bila vzpostaviti učinkovit sistem za merjenje aktivnosti LPS preko kompleksa MD-2/TLR4 v humani celični kulturi. Nato smo testirali snovi, za katere smo na podlagi strukturnega modela MD-2 predvidevali, da bi se lahko vezale na vezavno mesto LPS. Namen je bil identificirati substance, ki bi antagonizirale aktivacijo kompleksa MD-2/TLR4 z LPS. Dokazali smo, da kemoterapevtika taksol in taksoter zavirata LPS inducirano celično aktivacijo. Nadalje smo pokazali, da nekateri endogeni fosfolipidi lahko sprožijo vnetni odziv celic, kar bi lahko imelo vlogo pri aterosklerozi. Spojine, identificirane v diplomskem delu bodo pripomogle k boljšemu razumevanju mehanizma aktivacije MD-2/TLR4 in hkrati predstavljajo izhodišče za razvoj boljših antagonistov LPS, ki bi se lahko izkazala kot potencialna zdravila.

MEASURING OF ANTAGONISTS LPS MEDIATED SIGNALLING BY THE RECEPTOR COMPLEX MD-2/TLR4

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Lipopolysaccharide (LPS), a major component of the outer membrane of Gram-negative bacteria, stimulates a potent innate immune response in mammals that can result in septic shock and death. Cellular response to LPS is mediated by the receptor complex consisting of transmembrane Toll-like receptor 4 (TLR4) and MD-2. MD-2 is a secreted protein that interacts with the extracellular portion of TLR4 and binds directly LPS. Inhibition of the LPS-induced cell activation by specific antagonists could be an effective therapeutic approach for septic shock due to Gram-negative bacteria. Our first goal was in restoring an efficient system for measurement of LPS induced signal transducation by the receptor complex MD-2/TLR4 in human cell line. Secondly, we tested some molecules that according to the structural model of MD-2 could potentially bind to the LPS binding site. We tried to identify molecules that could antagonize LPS-induced activation of MD-2/TLR4. Our experiments have shown that two chemotherapeutic agents, taxol and taxotere respectively, had an inhibitory effect on the LPS induced cell activation. Furthermore we have demonstrated that some endogenous phospholipids, which are proposed to be important initiators of atherosclerosis, triggered inflammatory cell response. Molecules identified in this research not only increased our understating of underlying molecular mechanisms of MD-2/TLR4 activation, but could also serve as basis for the development of better LPS antagonists. They could be used as potential drugs.

PRETVORBE NEKATERIH DERIVATOV BICIKLO[2.2.2]OKTENA S HIDRAZINI

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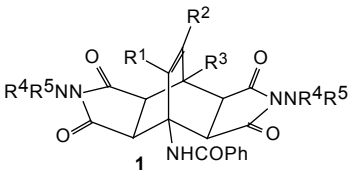
Svetovni napredek prinaša poleg blaginje tudi onesnaževanje okolja, zato se pri razvoju novih metod v organski sintezi vedno bolj upošteva aspekte ″zelene kemije″. Pri tem je mišljena predvsem uporaba negorljivih in netoksičnih topil ter obsevanje z mikrovalovi. Te aspekte sem tudi sam upošteval pri pretvorbah biciklo[2.2.2]oktenov s hidrazini, ki sem jih izvajal v vodi pod pogoji obsevanja z mikrovalovi. Ta sinteza produktov **1** se je izkazala za precej bolj učinkovito od klasičnega refluksa; reakcijski časi so se znatno zmanjšali, izkoristki reakcij zelo povečali, izolacija produktov je preprostejša (celo enostavnejša kot pri reakcijah, izvedenih brez topil), poleg tega pa so bili pri vseh reakcijah upoštevani aspekti “zelene kemije”. Posebej velja poudariti, da so celo sterično ovirani hidrazini (1,1-dimetil-, fenil- in 2-piridilhidrazin) pod mikrovalovi zreagirali z biciklo[2.2.2]oktenskim sistemom (te reakcije sicer pod refluksom v etanolu niso potekle). Tudi zato predstavlja opisana sinteza velik doprinos k ekološko sprejemljivejšim metodam v organski kemiji.



1

TRANSFORMATIONS OF BICYCLO[2.2.2]OCTENE DERIVATIVES WITH HYDRAZINES

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The global development bringing prosperity to the humankind is connected with the increasing environment pollution, causing growing awareness of the green chemistry principles. Use of nonflammable and nontoxic solvents and microwave irradiation is preferred. In my work I have tried to involve these aspects in the transformations of bicyclo[2.2.2]octenes with hydrazines. Comparing with ordinary reflux conditions, the reactions were successfully carried out in water under microwave irradiation producing **1** with significantly shorter reaction times, with increased yields and with simpler isolation procedures, incorporating the green chemistry principles. It is remarkable that also sterically bulky hydrazines (i.e. 1,1-dimethyl-, phenyl- and 2-pyridylhydrazine) reacted with bicyclo[2.2.2]octene systems (these reactions were in refluxing ethanol not successful). Described synthesis is an important addition to the development of eco-friendly methods in organic chemistry.

(1) M. Martelanc, K. Kranjc, S. Polanc, M. Kočevar *Green Chem*. **2005**, *7*, 737−741.

ELEKTROGENSKA TRANSFEKCIJA V ODVISNOSTI OD PONAVLJALNE FREKVENCE PULZOV IN ORIENTACIJE ELEKTRIČNEGA POLJA

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Elektrogenska transfekcija je neviralna metoda, pri kateri s pomočjo visokonapetostnih električnih pulzov vnašamo genski material v celice. Delovanje elektrogenske transfekcije temelji na elektroporaciji, metoda pa se je izkazala za uspešno pri vnosu genov v celice v *in vitro* ter *in vivo* pogojih. V tej nalogi smo raziskali, kako ponavljalna frekvenca in orientacija električnega polja vplivata na elektrogensko transfekcijo. Učinkovitost elektrogenske transfekcije smo ugotavljali z določanjem deleža transfektiranih celic in intenzitete fluorescence transfektiranih celic. Za transfekcijo smo uporabili plazmidno DNK (pEGFP-C1), ki izraža protein zelene fluorescence. Rezultati elektrogenske transfekcije kažejo, da delež transfektiranih celic pada z naraščanjem ponavljalne frekvence, pri frekvencah višjih od 0.2 Hz pa je odvisen tudi od orientacije električnega polja. Razlog za padec deleža transfektiranih celic s frekvenco je manjše preživetje celic po elektroporaciji pri teh frekvencah. Rezultati intenzitete fluorescence kažejo, da ima smer polja različen vpliv na odvisnost fluorescence od frekvence električnega polja. Intenziteta flourescence transfektiranih celic pri enosmernem električnem polju raste s frekvenco, medtem ko pri visokih frekvencah izmeničnega električnega polja pada. Spremembo intenzitete smo razložili s teorijo prehoda plazmidne DNK skozi celično membrano. Pokazali smo, da za plazmidno DNK obstaja energijska prepreka pred celično membrano, zaradi katere se DNK brez zunanjega električnega polja težko pritrdi na celično membrano. Na podlagi rezultatov lahko zaključimo, da je za uspešnejšo elektrogensko transfekcijo boljše izmenično polje, ker pri tem polju dobimo večji delež transfektiranih celic. Razlog za to je večja površina elektroporirane membrane, skozi katero lahko vstopi molekula DNK.

ELECTROGENE TRANSFECTION IN DEPENDENCY ON PULSE REPETITION FREQUENCY AND ELECTRIC FIELD ORIENTATION

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Electrogene transfection is a nonviral method used to transfer genes into living cells by means of high-voltage electric pulses. Electrogene transfection is therefore based on electroporation and it has been proven to be successful in *in vivo* and *in vitro* conditions. In this thesis we studied the influence of pulse repetition frequency and electric field orientation on the efficiency of electrogene transfection. The efficiency of electrogene transfection was determined by the fraction of transfected cells and the fluorescence intensity of transfected cells. The plasmid DNA (pEGFP-C1), which expresses green fluorescent protein (GFP), was used for transfection. The results of electrogene transfection show that the fraction of transfected cells decreases with the increase of the frequency. At frequencies higher than 0.2 Hz the fraction of transfected cells also depends on the field orientation. The decrease of cell transfection is most probably due to the decrease of cell viability at these frequencies. The results of fluorescence intensity show that at a given frequency fluorescence intensity depends on electric field orientation. For a direct electric field the fluorescence intensity increases with frequency, while for alternating electric fields it decreases. The change in intensity was explained using the theory of the transport of the plasmid DNA through the membrane. We proposed that there is an energy barrier, which enables the DNA to bind on the membrane without external electric field. On the basis of our results we can conclude that for the electrogene transfection the alternating electric field is more efficient than the direct electric field, because it results in a higher fraction of transfected cells.

UPORABA HIBRIDNIH NEVRONSKIH MREŽ ZA NAPOVEDOVANJE OBMOČJA NASTANKA IN KARAKTERIZACIJO MIKROEMULZIJ

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Mikroemulzije so bistri, termodinamsko stabilni koloidi, ki se uporabljajo kot nosilni sistemi za dostavo učinkovin. Poznavanje lastnosti mikroemulzij je bistvenega pomena za njihovo uporabo, zato smo za napovedovanje njihovih lastnosti skonstruirali dve hibridni nevronski mreži. Prvo smo uporabili za napovedovanje območja nastanka in karakterizacijo mikroemulzij s sestavo Tween 40®, Imwitor 308®, izopropilmiristat in voda. Dobili smo odlične rezultate z 90% pravilnostjo pri napovedi območja nastanka posameznih vrst mikroemulzij. Z drugo nevronsko mrežo smo izvajali avtomatsko direktno interpretacijo termogramov, posnetih z diferenčnim dinamičnim kalorimetrom (DSC). S proučevanjem vpliva velikosti in raznolikosti baze podatkov za učenje nevronske mreže smo dosegli 100% pravilno interpretacijo DSC termogramov.
Pravilno predvidena območja nastanka mikroemulzij in avtomatska interpretacija eksperimentalnih podatkov nam omogočata učinkovitejše in cenejše raziskave na področju mikroemulzij. V prihodnosti pa lahko podobne sisteme uporabimo tudi v druge namene, kot je naprimer proučevanje bližnjih infrardečih (NIR) spektrov v procesno analiznih tehnologijah (PAT).

USE OF HYBRID NEURAL NETWORK FOR PREDICTING MICROEMULSIONS FORMATION AND THEIR CHARACTERISTICS

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Microemulsions are transparent, thermodynamical stable colloids and are used as vehicles in drug delivery systems. Knowledge about their microemulsion characteristics are crucial for their use, and therefore we have constructed two hybrid neural networks for prediction of their properties. First one was used for predicting microemulsions formation and their characteristics with composition of Tween 40®, Imwitor 308®, isopropyl miristate and water. We have achieved excellent results with 90% correctness on different types of microemulsion formation. Second neural network was used for automatic direct interpretation of thermograms taken with differential scanning calorimeter (DSC). During learning database size and variability dependency testing we have increased precision of DSC thermogram interpretation to 100%.
Using high accuracy predictions and automatic data interpretation we can lower expenses and shorten research time on microemulsions investigation and make better predictions of their characteristics and usability. In future we can use similar systems for other purposes such as research of near infrared (NIR) spectra in process analysis technologies (PAT).

PRIMERJAVA FIZIKALNO-KEMIJSKIH LASTNOSTI HIDRATOV K-0805

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Trdne snovi lahko kljub isti kemični sestavi obstajajo v različnih kristalnih oblikah, polimorfih. Kadar se v kristalno rešetko vežejo molekule topila, govorimo o solvatih, pri vezavi vode gre za hidrate.
Proučevali smo dve obliki K-0805, in sicer monohidrat in seskvihidrat. Ugotovili smo, da se monohidrat najhitreje raztaplja in ima najvišjo topnost, ki pa je težko določljiva, ker pri raztapljanju v fosfatnem pufru pH 9 prehaja v termodinamsko stabilnejši dihidrat. Seskvihidrat, ki je ostal nespremenjen, ima nižjo topnost, najnižjo topnost pa ima dihidrat, kar potrjuje pravilo, da je hidrat z največjo stopnjo hidratacije najmanj topen. Iz podatkov topnosti pri različnih temperaturah smo narisali van`t Hoffov diagram za oba hidrata ter iz naklona premice izračunali navidezno entalpijo raztapljanja. Iz presečišč premic na van`t Hoffovem diagramu smo določili teoretično temperaturo prehoda K-0805 monohidrata v seskvihidrat. Temperaturo prehoda med obema oblikama smo določili tudi s pomočjo izračuna proste Gibbsove entalpije. Pri določevanju entalpije raztapljanja z mikrokalorimetrijo smo opazili skoraj linearno naraščanje le-te s temperaturo. Iz naklona premice smo določili razliko molarne toplotne kapacitete pri konstantnem tlaku ($\Delta c_p = (c_p)_{prod} - (c_p)_{reakt}$). Z DSC analizo smo določili temperaturo tališča in talilno entalpijo za brezvodno obliko K-0805, ki smo jo pripravili z izotermalnim sušenjem monohidrata ali seskvihidrata. S termogravimetrično analizo smo določili vsebnost vode v obeh oblikah K-0805.

COMPARISON OF PHYSICOCHEMICAL PROPERTIES OF K-0805 HYDRATES

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Solid substances with the same chemical structure can exist in different crystal forms, called polymorphs. Solvates are structures with solvent molecules incorporated in crystal lattice form. When these are water molecules, hydrates are formed.
In our research we studied K-0805 monohydrate and sesquihydrate. Monohydrate has the highest dissolution rate and the highest solubility, which is difficult to determine, because it converts to thermodynamically more stable dihydrate during dissolution in phosphate buffer with pH 9. Sesquihydrate does not convert into more stable form and has lower solubility, while the lowest solubility has dihydrate. This is in accordance with the rule that the most hydrated form has the lowest solubility. Using the solubility data for both hydrates we prepared van`t Hoff plot and calculated solution enthalpy from the slope of the curve. From interception of both curves we determined theoretical temperature of transition of monohydrate to sesquihydrate. We also determined temperature of transition by calculating Gibbs free enthalpy. Solution calorimetry results show that the solution enthalpy increases almost linear with temperature. From the slope of the curve we determined the difference in molar heat capacity at constant pressure ($\Delta c_p = (c_p)_{prod} - (c_p)_{reakt}$). Using DSC analysis we determined melting temperature and melting enthalpy for anhydrous form of K-0805, which was prepared by isothermal drying of monohydrate or sesquihydrate. The amount of water was also determined in both hydrates by thermogravimetric analysis.

CITRONKA (*Lippia citriodora* Kunth.)

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V raziskovalni nalogi sva v teoretičnem delu podrobno analizirali vse ključne elemente citronke (izvor imena, razširjenost, primerno podnebje, rastišče, gojenje, zdravilni učinki, …) ter še posebej njeno eterično olje. Slednje naju je pritegnilo, ker je bilo leta 2002 v okviru evropskega parlamenta uvrščeno na listo kemikalij, ki so v kozmetičnih izdelkih prepovedane, kljub temu pa sva ga zasledili kot sestavino le teh.
V eksperimentalnem delu sva na osnovi postopka vodne destilacije pridobili citronkino eterično olje ter s pomočjo plinske kromatografije določili njegovo sestavo. Ugotovili sva, da citronkino eterično olje vsebuje največ citrala in geraniola, ki sta fotosenzibilna in zato lahko ob stiku z UV svetlobo povzročata poškodbe kože. V reviji Hagers Handbuch der Pharmazeutische Praxis pa sva zasledili podatek, da obstaja še ena vrsta eteričnega olja iz citronke, in sicer Verbena absolute. Uporaba tega je v kozmetičnih izdelkih dovoljena, če njegov delež ni večji od 0,2 %. V omenjeni nemški farmacevtski reviji sva zasledili podatek, da Verbena absolute lahko pridobimo s pomočjo alkoholne destilacije z etanolom. To sva opravili, vendar eteričnega olja s pomočjo Clevenger aparata, ki sva ga uporabljali, nisva mogli pridobiti, saj je eterično olje citronke v etanolu topno. V hrvaški farmacevtski reviji Farmaceutski glasnik pa sva zasledili podatek, da je vrsta citronkinega eteričnega olja oziroma njegova sestava odvisna od kemotipa rastline.
Verbena absolute naj bi tako pridobivali iz citronke, gojene v Franciji, ki vsebuje manj citrala, ki je sicer glavni razlog za prepoved uporabe citronkinega eteričnega olja.

CITRONKA (*Lippia citriodora* Kunth.)

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Our research paper is divided into two parts. In theoretical part we were searching and analyzing information about Verbena that we found in books from its name origin, place of growth, suitable climate for its growth, possible usage in alternative medicine, … to its eteric oil – verbena oil. When we found out that in 2002 the European Parliament listed verbena oil on the list of chemicals that are prohibited to use in cosmetic products, but still products containing verbena oil can be found in shops, we decided to analyze verbena oil.
In the experimental part we used special techniques such as water distillation and gas chromatography to prepare and analyze verbena oil. We found out that verbena oil contains a high percent of geraniol and citral, two chemical substances which are photo sensible. That means that they can react with UV light and cause serious skin damages. But on the other hand as said in Hagers Handbuch der Pharmazeutische Praxis there exists another use in cosmetics, if its percentage in the product is less than 0,2 %. We found out that this type of verbena oil is named Verbena absolute and can be prepared with using the distillation method with ethanol. We performed distillation as described, but there was no result. Later we discovered that there can be two possible reasons for unsuccessful experiment. Firstly the unsuitable distillation apparatus and secondly unsuitable type of verbena. In Croatian pharmaceutical magazine Farmaceutski glasnik was said that fragrances of verbena oil depend on chemotype of the plant.
Verbena absolute is prepared from verbena that contains less citral which is the main reason for the prohibition of verbena oil in cosmetic products. This kind of verbena grows only in France.

KVALITETA CVIČKA RAZLIČNIH PROIZVAJALCEV V PRIMERJAVI Z NEKATERIMI RDEČIMI VINI IN NJIHOVA ZDRAVILNA MOČ

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Cviček je na Slovenskem čedalje bolj cenjena pijača. Je suho, lahko, svetlo rdeče vino brez izrazite trpkosti. V dobrih vinogradniških legah je najprimernejša sestava sort v razmerju: 70% žametovke, 20% kraljevine in 10% frankinje in nekaj odstotkov laškega rizlinga. Fermentacija grozdnega soka v vinu je kompleksen mikrobiološki proces, ki obsega postopen razvoj in interakcije med različnimi mikroorganizmi, kot so kvasovke, bakterije in nitaste glive. Med omenjenimi mikroorganizmi imajo kvasovke zdaleč največji pomen, saj imajo sposobnost pretvorbe sladkorjev grozdja v etanol, ogljikov dioksid ter v ostale pomembne metabolite v nižjih koncentracijah. Včasih dvoje vin iste sorte in istega vinograda pokaže različno sestavo, če je vrelo različno ali v drugačnih sodih.
Opravile smo analize mošta in vina, da bi ugotovile vrednosti določenih parametrov. Posluževale smo se naslednjih metod: določanje kislin z nevtralizacijsko titracijo, merjenje suhe snovi mošta z namiznim refraktometrom, določanje pH mošta, določanje sladkorja v moštu z Öechslerjevo moštno tehtnico, določanje alkohola v vinu z ebulioskopsko metodo, določanje skupnih kislin v vinu, določanje žveplovega dioksida po Ripperju, določanje količine pepela v vinu in določanje žveplove kisline z jodometrično titracijo. Kot glavno metodo za ugotavljanje bakteriocidnega oziroma bakteriostatičnega delovanja smo uporabile difuzijski antibiogram.

THE QUALITY OF CVIČEK BY DIFFERENT PRODUCERS AND ITS HEALTHY EFFECTS IN COMPARISON WITH SOME OTHER RED WINES

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Cviček has become a more and more respected wine sort. It is dry, light red wine without distinctive bitterness. In vineyards with appropriate geographical position the most proportionate combination of sorts is: 70% of žametovka, 20% of kraljevina and 10% of frankinja and a few percents of laški rizling. The fermentation of grape juice into wine is a complex microbiological process, which includes gradual progress and interactions between different microorganisms, such as yeasts, bacteria and fungus. Among the above mentioned organisms by far the most important are the yeasts, because they have the ability of transformation of grape sugar into ethanol, carbon dioxide and other important metabolites in lower concentrations. Sometimes it is possible for two wines from the same vineyard and the same sorts to show different values if it is fermented differently and in different containers.
We have analyzed must and wine, using the following methods: determination of acids with neutralization titration, measurement of dry substances in must with refractometer, determination of must pH, determination of must sugar with Öechsley must scale, determination of alcohol degree in wine with ebullioscope method, determination of collective acids in the wine, determination of sulfuric dioxide round Ripper, determination of amount of ash in wine, determination of sulphuric acid iodometric titration. As the main method for determination of bactericidal and bacteriostatic process we used a diffuse antibiogram.

ODPORNOST BAKTERIJ RODU *Bacillus* PROTI RAZMERAM V EKSTREMNIH OKOLJIH

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Bakterije rodu *Bacillus* so prisotne v širšem naravnem okolju. Najdemo jih v vseh vrstah hrane, smeteh, kompostu, iztrebkih, usedlinah in vodi. Za rast potrebujejo organske snovi, pridobljene iz rastlinskih in živalskih virov. Večinoma niso patogene, kljub temu nekatere vrste povzročajo bolezni ljudi, rastlin in živali ter zastrupljajo hrano. V industriji se bakterije rodu *Bacillus* uporabljajo za pridobivanje antibiotikov, insekticidov ter industrijskih encimov. Značilnost tega rodu je tvorba endospor (stadij celice z visoko odpornostjo). V taki obliki lahko celice preživijo ekstremne razmere, kot so vrenje in UV sevanje.
V raziskovalni nalogi smo preučevali odpornost spor *Bacillus sp*. na toploto in UV žarke. In sicer smo z obdelovanjem spor z različnimi preparati, kot so klorovodikova kislina, kovinski ioni (Zn^{2+}, Ca^{2+}, Mg^{2+}) in mešanica mineralov imenovana mikrominerali, želeli ugotoviti principe za doseg najvišje in najnižje odpornosti spor na UV žarke in toploto. 0,01 M klorovodikova kislina je ustrezno sredstvo za zmanjševanje odpornosti spor in vzdrževanje konstantne biološke aktivnosti.
Mikrominerali so najboljša kombinacija mineralov za izboljšanje stabilnosti spor, saj so zvišali odpornost na UV sevanje za več kot stokrat.

RESISTANCE OF MEMBERS OF THE GENUS *Bacillus* TO EXTREME ENVIRONMENTAL CONDITIONS

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The bacteria of the *Bacillus* species are widespread in the nature. They could be in all sorts of foods, excrements, garbage, compost piles, earth sediments and water. As a substrate they need organic material derived from plant and animal sources. Mostly they are not pathogenic although certain strains or species are causing diseases with the humans, animals and plants (the pathogens); they are spoiling our food and could cause poisonings (food spoilers). *Bacilli* are well known producers of certain antibiotics, insecticides and industrial enzymes. Their common characteristic is the endospore formation. Spores are highly resistant resting stadium of the cell. Therefore they could survive boiling and UV irradiation.
We studied heat and the UV resistance of the *Bacillus sp*. spores. We treated spores of the *Bacillus sp*. with different substances including hydrochloric acid, metal cations (Zn^{2+}, Ca^{2+}, Mg^{2+}) and combinations of micro-minerals to define environmental conditions (cultivation and storage medium) for the highest and for the lowest UV and heat resistance. 0,01M hydrochloric acid has been applied to reduce the spore resistance and to keep their biological activity constant.
We defined the best combination of different minerals – micro-mineral solution that improved stability of the spore population against the UV irradiation for more than hundred times.

ANTACIDI

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Izdelali smo anketni vprašalnik in izvedli anketo o poznavanju, vrstah in uporabi sredstev za zmanjšanje želodčne kisline oz. za povišanje pH vrednosti v želodcu. V anketi največkrat omenjene antacide (Rupurut, Rutacid, Rennie, Gastal, Kompensan) in Ranital ter druge pripravke (sok rdeče pese, rdeče vino, presni krompirjev sok, Donat in soda bikarbona) smo uporabili v eksperimentalnem delu. Raziskovali smo, kako bi s pomočjo meritev spremembe kislosti in bazičnosti primerjali njihovo aktivnost v želodcu. Izdelali smo navodila za vajo, s katero lahko prikažemo reaktivnost antacidov, sadnih in zelenjavnih sokov z 0,1 M klorovodikovo kislino. Meritve smo opravili s pH metrom, vezanim na računalnik, ki nam je izrisal tudi krivulje spreminjanja pH vrednosti v odvisnosti od časa. Iz teh krivulj sklepamo, da so učinki snovi različni, nekateri hitri, vendar kratkotrajni, drugi postopni in trajajo daljši čas, nekatera sredstva proti bolečinam v želodcu niso antacidi. Ugotovili smo, da vpliva na znižanje želodčne kisline tudi mineralna voda, presni krompirjev sok in sok rdeče pese.

ANTACIDS

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We wanted to find out what people know about the existence of various substances which reduce the quantity of gastric acid (for increasing the pH level). We also wanted to find out how they use these substances. For these reasons we designed and carried out a questionnaire. The antacids which were most often mentioned by those who completed the questionnaire were Rupurut, Rutacid, Rennie, Gastal, Kompensan and some natural substances, such as beetroot juice, red wine, potato juice, mineral water Donat and sodium bicarbonate. These antacids and natural substances were used in our laboratory experimental work. The reactivity of the above mentioned substances in the stomach was compared by means of measuring their change in the acidity and alkalinity. We designed follow-up instructions for the experiment with 0,1 M hydrochloric acid, by which it would be possible to show the degree of reactivity of individual antacids and individual fruit and vegetable juices. The measurements were performed by a pH meter connected to a computer which recorded the change of pH levels in correlation to time by drawing graphs. On the basis of the given figures it was possible to infer that the individual substances and individual antacids used in the experiment had shown different effects. Some substances showed immediate but short-term effects, others showed gradual but longer-lasting effects. However, some substances against stomach ache were not antacids. Results of the research proved that not only the presence of antacids, but also the presence of natural substances, such as mineral water, potato juice and beetroot juice can result in the reduction of the quantity of gastric acid.

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