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BOOK of ABSTRACTS

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BOOK OF ABSTRACTS

ORAL PRESENTATIONS

Section: Cellular biochemistry

Deciphering endoplasmic reticulum associated protein degradation: EDEM proteins

Stefana M Petrescu *
Institute of Biochemistry, Romanian Academy, Splaiul Independentei 296, Bucharest, Romania
**email: stefana.petrescu@biochim.ro*

Introduction: Endoplasmic reticulum associated protein degradation, ERAD, is a highly regulated process that clears the crowded ER lumen of polypeptides with fatal folding errors, leaving the ER in a folding competent state. EDEM proteins are able to recognize some misfolded glycoproteins and to trim the Man9 N-glycans in order to target the glycoproteins to degradation by proteasomes. Despite many advances in the field and the validation of a number of mutants that are EDEM substrates there is still unclear what is the physiological role of this family of proteins and whether there are endogenous proteins targeted by EDEM during ERAD. EDEM family includes three proteins, EDEM-1, EDEM-2 and EDEM3, highly conserved from *C.elegans* to mammalian. We addressed te role of EDEMs in C.elegans in order to understand their physiological role in an organism. We also aimed to identify EDEMs interactors in mammalian sysytems and endogenous proteins that are targeted to degradation by EDEMs. [1, 2, 3].

Materials and methods: We used CRISPR/Cas technology to obtain cell lines depleted in genes involved in ERAD. *C elegans* was used to investigate the ERAD at an organismal level. To identify interactors and ERAD substrates we used mass spectrometry based proteomics.

Results: Using the model organism C.elegans, we found that there are ditinctive differences in the roles of the three EDEMs, with EDEM2 validated as physiologically crucial in maintaining protein homeostasis. The other two EDEMs are required to protect the organism during acute ER stress. We also report the discovery of a number of endogenous substrates for EDEM2 whose half-life deetrmined using SILAC high performance mass spectrometry was regulated by EDEM2. EDEM3, one of the most active EDEM mannosidase has four consecutive modules that we were able to model and address their individual roles. We also identified a particular sensitivity of EDEM3 towards the ER redox environement, suggesting a mechanism involving reaarrangements of disulfide bridges.

Conclusions: EDEM proteins are involved in the quality control of endogenous proteins in mammals and the role of the three proteins is significantly different under physiological ER stress conditions.

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New molecular mechanisms involved in melanoma cell resistance to nutritional and chemotherapeutic stress

Filimon Anca[a,*], **Negroiu Gabriela**[b]

a) b) Institute of Biochemistry of Romanian Academy, Splaiul Independentei nr. 296, Bucharest, Romania
**email:* anca_m_filimon@yahoo.com

Introduction: During tumor development cancer cells are subjected to various stress conditions due to high proliferation rates, limited levels of oxygen, glucose, amino acids or the presence of reactive oxygen species. Tumor expansion and metastasis can be successful only if cancer cells can adapt to these stressful conditions through activation of signaling cascades and transcriptional programs which determine the expression of the necessary adaptive genes. Activating transcription factor 4 (ATF4) is the master regulator of cellular stress responses triggered by multiple signaling pathways that form the integrated stress response (ISR). The phosphorylation of the alpha subunit of eukaryotic translation initiation factor 2 (eiF2α) on serine 51 is the core event of the ISR that leads to a marked reduction in global protein synthesis while allowing the translation of selected genes including ATF4. Besides the canonical induction by ISR recent studies have shown that ATF4 is activated by the mechanistic target of rapamycin complex 1 (mTORC1) as a part of an anabolic program initiated by pro-growth signals (1). While translational regulation of ATF4 is one of the main mechanisms for ATF4 expression, transcriptional control is also important for ATF4 mediated cellular processes (2). Besides stress conditions generated by a rapid tumor growth and anabolic processes, ATF4 is also induced by chemotherapeutic stress and can be a major player in tumor cell resistance to chemotherapy (3). Dopachrome tautomerase (DCT) is an enzyme involved in the distal step of melanin biosynthesis in melanocytes and melanoma cells and has been shown to reduce melanoma cells sensitivity to oxidative stress and protect cells from UV or X ray induced apoptosis. So far, the molecular mechanisms through which DCT modulates cellular stress responses have not been elucidated. Moreover, DCT expression assessed in melanoma FFPE specimens has been associated with unfavorable clinical parameters (ulceration and a high number of mitoses) and biomarkers indicative of metastatic progression (4).

Materials and methods: melanoma primary and metastatic cell lines (WM35, WM39, MJS, SKMel23, SKMel28), cell culture media, supplements and sterile plasticware, siRNAs, CRISPR/Cas9, LC/MS, RT-qPCR, Western Blot.

Results: ATF4 and DCT expressions are correlated in MJS growing cell cultures at protein and mRNA levels and respond to variations in the concentrations of non essential aminoacids.

Downregulation of DCT expression through siRNAs or CRISPR/Cas9 determines a marked reduction in the expression of ATF4 and its target gene asparagin synthetase (ASNS) at protein and mRNA levels.

DCT protein levels are upregulated in BRAF mutated V600E melanoma cells treated with Vemurafenib or in BRAF WT cells where BRAF expression is silenced with siRNAs.

Conclusions: DCT is a novel protein associated with ATF4 expression either as part of the ISR or in anabolic processes controlled by mTOR signalling. DCT is a novel regulator of ATF4 and its target gene ASNS at mRNA and protein levels in multiple BRAF WT and BRAF mutated melanoma cell lines. DCT is upregulated as a result of signalling blocade on RAS-RAF-MAPK-ERK pathway and is a novel candidate player in the resistance to chemotherapy through regulating ATF4 expression levels. ***The decipheration of DCT-ATF4 axis is important for a better understanding of melanoma molecularity and possibly for development of novel therapeutical approaches in some tumor subtypes.***

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Curcumin reverses the irinotecan acquired resistance in colorectal cancer cells, in vitro

Cruceriu Daniel [a,b*]**, Gavrilaș Laura** [c] **, Sava Oana** [b]

a) Department of Molecular Biology and Biotechnology, "Babes-Bolyai" University, 5-7 Clinicilor Street, Cluj-Napoca, Romania;
b) Department of Genetics, Genomics and Experimental Pathology, The Oncology Institute "Prof. Dr. Ion Chiricuta", 34-36 Republicii Street, Cluj-Napoca, Romania;
c) Department of Bromatology, Hygiene, Nutrition, "Iuliu Hatieganu" University of Medicine and Pharmacy, 23 Marinescu Street, Cluj-Napoca, Romania
**email: daniel.cruceriu@ubbcluj.ro*

Introduction: A major problem in the management of colorectal cancer (CRC) is represented by the tumor acquired resistance to conventional chemotherapy, including irinotecan, a compound usually administrated in advanced CRC, as a first- or second-line chemotherapeutic drug. Specific plant-derived compounds have the potential to resensitize chemoresistant cancer cells to certain drugs. Curcumin, the major bioactive compound found in *Curcuma longa* is one such promising compound. Therefore, the purpose of this study was to assess curcumin's capacity to reverse the acquired resistance to irinotecan of CRC cells and provide novel insights into the cellular and molecular processes associated with it.

Materials and methods: An irinotecan-resistant cell line (DLD1_IRI-R) was obtained through successive exposures of DLD1 cells to increasing concentrations of irinotecan, until cells would survive and proliferate in culture medium containing the drug at a concentration of 20 µM, the concentration at which irinotecan is found in the blood stream of CRC patients. The MTT assay was used to evaluate the sensitivity of both cell lines to irinotecan and curcumin, administered alone or in combination at different concentrations, at 48h after exposure. The nature of the interaction between the two compounds was determined by the Chou-Talalay method, whereas curcumin's capacity to reverse the irinotecan-acquired resistance was evaluated based on the Reversal Fold (RF) parameter. Flow-cytometry was used to assess apoptosis, whereas clonogenicity assays were used to monitor proliferation in all experimental settings. Whole-genome transcriptional profiling of the irinotecan-sensitive, irinotecan-resistant and curcumin-treated irinotecan-resistant cell lines was performed by microarray technology, whereas the functional analysis of the data was performed in IPA (Ingenuity Pathway Analysis) and GESA (Gene Set Enrichment Analysis).

Results: DLD1_IRI-R cells were 7.17X more resistant to irinotecan than the parental cells. Based on the whole transcriptome data, as well as the clonogenicity assays, one of the main mechanisms by which CRC cells acquire resistance to irinotecan is by slowing down the cell cycle, several molecular pathways involved in proliferation being inhibited. Interestingly, the resistance to curcumin also increased (3.2X) in the irinotecan-resistant cell line, suggesting the activation of more general drug-induced resistance mechanisms. When a combinatorial treatment of irinotecan in successive concentration and curcumin at a constant low concentration (IC_{10}) was administered on DLD1_IRI-R cells, a significant decrease in the irinotecan specific IC_{50} was observed, demonstrating a resistance reversal fold of 3.74X. Furthermore, the combined treatment was characterized by synergistic effects on the resistant cell line according to the Chou-Talalay analysis (CI<1) for every tested combination of concentrations, both in experiments with a constant-dose ratio and in combinations of irinotecan with curcumin at IC5, IC10, IC15 or IC20. The observed synergistic effects are most likely due to the different mechanisms by which the compounds affect cells: irinotecan triggers cell cycle arrest, while curcumin induces apoptosis, as demonstrated by flow-cytometry. Besides inducing apoptosis, curcumin also reactivated several signaling pathways which promote proliferation and triggered the inhibition of several genes known to be involved in drug efflux, increasing the overall sensitivity of the tumor cells.

Conclusions: Curcumin might be capable of reversing the acquired resistance to irinotecan in CRC cells by inducing apoptosis, retriggering cell proliferation and sensitizing cells to irinotecan, whereas the combinatorial therapy could increase the conventional treatment efficacy.

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GPR75, an unusual and mesmerizing novel therapeutic target

Teodora Stratulat [a, c], Ramona Tecucianu [a], Cristin Coman [b], Aura Ionescu [a], Cosmin Trif [a],
Anghel S. Andreea [a], Rodica Badea [a], Alexandru Babes [c], Stefana Petrescu [d], Sorin Tunaru [a,*]

a) Cell Signalling Research Group, Institute of Biochemistry of the Romanian Academy, Splaiul
Independentei 296, Bucharest, Romania;
b) National Institute of Research and Development for Microbiology and Immunology "Cantacuzino",
Splaiul Independenței, nr. 103, București, Romania;
c) Department of Anatomy, Physiology, and Biophysics, Faculty of Biology, University of Bucharest,
Splaiul Independenţei 91-95, 050095 Bucharest, Romania;
d) Molecular Cell Biology Department, Institute of Biochemistry of the Romanian Academy, Splaiul
Independentei 296, Bucharest, Romania;
**email: sorin.tunaru@googlemail.com*

Introduction: GPR75 belongs to the largest superfamily of membrane receptors, G-protein coupled receptors (GPCRs). The particularity of GPR75 is that no ligand or biological roles have been clearly assigned to it, despite several studies pointing to its implication in the regulation of human and mouse metabolism [1, 2]. In mouse and humans, GPR75 is highly expressed in brain, pancreas, liver and retina. Large scale human exome analysis associated GPR75 presence with increased sensitivity of individuals to obesity [1].

Materials and methods: HEK293T, COS-7, HeLa cell lines were transiently transfected with plasmids containing the cDNA of human GPR75, muscarinic acethyl-choline receptors (hM1-hM5) and genetic probes sensitive and specific for intracellular calcium and cAMP. Forty-eight hours after the transfection, cells were loaded with HBSS buffer containing luciferases and determination of agonists-induced messenger accumulation was performed by using a 96-well plate reader (Flexstation 3, Molecular Devices). Phylogenetic analyses were carried out by using MEGA11 software. GPR75 deficient mice were generated by homologous recombination in ES cells. Groups of wild-type and GPR75 deficient mice were subjected to high-fat diet for more than six month. Body weight, food and water consumption was recorded on a daily base.

Results: Our results indicate that GPR75 is an ancient receptor that resembles no sequence similarity to any known GPCR. Studies performed by expressing human GPR75 in HEK293T and other cell lines demonstrated a potential role in the modulation of other GPCRs and ion channels, heterologously or endogenously expressed. Cellular biochemistry assays identified several unusual interacting proteins that contribute to GPR75 expression at the cell surface. To examine GPR75 role *in vivo*, mouse model of *Gpr75* gene deficiency was generated and analyzed. The results proved that GPR75 receptor has important metabolic roles as evidenced by its contribution in models of diet-induced obesity and type-2 diabetes.

Conclusions: At cellular level, GPR75 appears to modulate the function of specific targets by an yet-to-be-identified mechanism. *In vivo*, GPR75 seems to play major roles in the regulation of metabolism, being a significant factor in obesity and diabetes.

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New cell-penettrating peptides with antimicrobial properties

Dana Maria Copolovici [a*], Andreea Gostăviceanu [a,b], Carmen Popa [a,b], Cristian Moisa [a],
Andreea Ioana Lupitu [a], Oana Gavriliuc [a,c,d], Florina Bojin [a,c,d], Lucian Copolovici [a]

a) "Aurel Vlaicu" University of Arad, Faculty of Food Engineering, Tourism and Environmental Protection and Institute of Technical and Natural Sciences Research-Development-Innovation of "Aurel Vlaicu" University, 310010, Elena Dragoi Street, nr. 2, Arad, Romania
b) University of Oradea, Biomedical Sciences Doctoral School, Universității Street, Nr.1, 410087, Oradea, Romania
c) "Victor Babes" University of Medicine and Pharmacy, Department of Functional Sciences, Immuno-Physiology and Biotechnologies Center, 300041 Timisoara, Romania
d) Timis County Emergency Clinical Hospital "Pius Brînzeu" Timisoara, Center for Gene and Cellular Therapies in the Treatment of Cancer—OncoGen, No. 156 Liviu Rebreanu, 300723 Timisoara, Romania
**email: dana.copolovici@uav.ro*

Introduction: Modern lifestyles and longer lifespans necessitate treatment for cancer, neurological diseases, and viral infections [1,2]. Thus, innovative, effective, and reliable methods with higher target cell specificity, low cost, and little impacts on healthy tissues are needed. Peptide-based systems could deliver drugs and genes. Cell-penetrating peptides (CPPs) are small molecules that may cross cell plasma membranes and carry cargo (drugs, nucleic acids, imaging agents, etc.) into the cytosol or nucleus. They are chemically changeable, stable, and non-immunogenic molecules.

Materials and methods: Solid-phase peptide synthesis (SPPS) and the labile fluorenylmethyloxycarbonyl group (Fmoc chemistry) strategy with solid Rink-amide resin were used to synthesize six peptides, three without stearyl moiety group, and three stearylated homologous peptides. The peptides were purified by preparative HPLC and their purity was determined using analytical HPLC and MALDI-TOF-MS analysis. Minimum inhibitory concentration (MIC) values were used to evaluate the antibacterial efficacy of the newly obtained peptides. Following Wiegand et al.'s (2008) published technique, the MIC of the peptides was determined in two bacteria (*Staphylococcus aureus* and *Klebsiella pneumoniae*) using the broth microdilution method. The cytotoxicity, cell proliferation and cellular metabolic activity was determined by microscopy and Alamar Blue assay at divers concentrations.

Results: Six peptides, named CPP-1 – CPP-6, were obtained. The antimicrobial activity against *Staphylococcus aureus* and *Klebsiella pneumoniae* is depended both on the peptides' sequence and peptides concentration. The majority of the peptides are not toxic in MDA-MB-231 and MCF-7 breast cancer cell lines, while all six peptides are not toxic on mesenchymal stem cells.

Conclusions: Designing peptides that can, among other things, break through the cell membrane or pass biological barriers, stay in circulation longer, and are not toxic or immunogenic to humans is crucial for the rapid development of peptide-based therapeutic systems.

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Iron oxide nanoparticles carried by probiotics in iron absorption

Oana Lelia Pop [a,b,c] *, Călina Ciont [a,b,c], Dan Cristian Vodnar [a,b,c] and Amalia Mesaros [d]

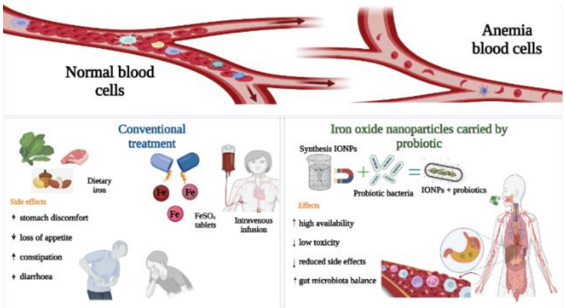
a) Department of Food Science, University of Agricultural Sciences and Veterinary Medicine, 400372, Cluj-Napoca, Romania;
b) Institute of Life Sciences, University of Agricultural Sciences and Veterinary Medicine, Calea Mănăştur 3–5, 400372 Cluj–Napoca, Romania;
c) Molecular Nutrition and Proteomics Laboratory, Institute of Life Sciences, University of Agricultural Sciences and Veterinary Medicine, 400372, Cluj-Napoca, Romania;
d) Physics and Chemistry Department, C4S Centre, Technical University of Cluj-Napoca, 28 Memorandumului Street, 400114 Cluj-Napoca, Romania;
**email:* oana.pop@usamvcluj.ro

Introduction: Recently, newly generated iron oxide nanoparticles (IONPs) carried by probiotics have been recommended as innovative iron supplements due to their low reactivity and high bioavailability compared to conventional anemia treatments [1]. Due to the probiotic's capacity to connect with the intestinal walls, IONPs-bacteria incorporate into the enterocyte, where nanoparticles are given, providing an adequate iron content [2]. The present study aimed to investigate the beneficial effect of IONPs with probiotics (average size 10 nm) as new iron supplements for iron deficiency in anemic rats compared with the conventional treatment ($FeSO_4$).

Materials and methods: The research included a comprehensive set of tests, including *in vitro* MRI, electronic microscopy investigations on the CaCO2 human cell line after treatment, and magnetic examination of the various rat tissues following administration of IONPs and IONPs with probiotics. Also, the restoration of healthy levels of blood parameters and iron-related protein expressions were investigated to confirm the efficiency of this material as a new drug for anemia.

Results: The efficiency of IONPs for the treatment of anemia was sensibly higher when nanoparticles were incorporated into the probiotic bacterium *Lactobacillus fermentum* than the conventional treatment ($FeSO_4$). Plasma iron and hemoglobin, intestine expression of divalent metal transporter 1 (DMT1) and duodenal cytochrome B (DcytB), as well as hepatic expression of the hormone hepcidin, was fully restored to healthy levels after administration of IONPs with probiotic.

Conclusions: The collective analysis of results points out that *L. fermentum* is an excellent carrier to overcome the stomach medium and drive IONPs to the intestine, where iron absorption occurs.



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The use of functionalized liposomes and extracellular vesicles as drug delivery systems with high cancer-targeting efficacy

Valentin Florian Rauca [a*], **Laura Patras** [a], **Giorgiana Negrea** [b], **Emilia Licarete** [a], **Alina Porfire** [c], **Lucian Barbu** [d], **Lavinia Luput** [a], **Alina Sesarman** [a], **Manuela Banciu**[a*]

a) Department of Molecular Biology and Biotechnology, and Center of Systems Biology, Biodiversity and Bioresources, Faculty of Biology and Geology, Babes-Bolyai University, Cluj-Napoca, Romania
b) Doctoral School in Integrative Biology, Faculty of Biology and Geology, Babes-Bolyai University, Cluj-Napoca
c) Department of Pharmaceutical Technology and Biopharmaceutics, Faculty of Pharmacy, University of Medicine and Pharmacy "Iuliu Hatieganu", Cluj-Napoca, Romania
d) ˝C. Craciun” Electron Microscopy Center, Faculty of Biology and Geology, “Babes-Bolyai” University, Cluj-Napoca, Romania
**email: valentin.rauca@ubbcluj.ro*

Introduction: To be considered efficient, Nanoparticle-based Drug Delivery Systems (NDDSs) face tremendous challenges related to toxicity, controlled release, stability, physical barrier penetration, clearance, and finally, enrichment at tumor sites in order to elicit desired therapeutic effects. Increasingly more studies focus on designing engineered nanoformulations with specific properties related to molecular level pathogenesis [1]. Among the most efficient delivery systems developed by our group are tumor cell-derived extracellular vesicles “sterically stabilized” with poly(ethylene glycol) (PEG) and loaded with doxorubicin (PEG-EV-DOX) to selectively target melanoma cells, and long circulating liposomes functionalized with IL-13 to ensure simvastatin (IL-13-LCL-SIM) or prednisolone (IL-13-LCL-PLP) drug delivery to tumor microenvironment cells such as tumor associated macrophages (TAMs).

Materials and methods: We used spectrofluorimetric and fluorescence microscopy methods to assess cellular uptake of our rhodamine-tagged engineered nanoformulations. To assess the antiproliferative potential of the drug-loaded delivery systems we used ELISA BrdU-colorimetric immunoassay. The antitumor effect of our NDDSs was investigated on C57BL6 mice bearing B16.F10 s.c melanoma tumors and molecular mechanisms with regard to major hallmarks of tumor progression (angiogenesis, oxidative stress, resistance to apoptosis, invasion) were surveilled by protein array, WB, HPLC and IHC.

Results: Experimental data has shown that PEG-EV-DOX were more efficient to inhibit B16.F10 murine melanoma growth than clinically applied long-circulating liposomal DOX (LCL-DOX) and reduced significantly melanoma aggressiveness and tumor angiogenesis. Furthermore, both functionalized liposomal formulations (IL-13-LCL-SIM, IL-13-LCL-PLP) showed higher antitumor potential than their nonfunctionalized counterparts (LCL-SIM, LCL-PLP). Thus, we proposed novel sequential or simultaneous administration therapies based on PEG-EV-DOX to precisely target melanoma cells and on IL-13-LCL-SIM or IL-13-LCL-PLP to ensure targeting of tumor microenvironment cells such as tumor associated macrophages (TAMs) [2], [3].

Conclusion: The novel NDDSs showed high efficiency in potentiating antitumor effects of all chemotherapeutic agents tested. The drug delivery strategy based on combined active targeting of both cancer cells and immune cells was able to induce a potent antitumor effect by disruption of the reciprocal interactions between TAMs and melanoma cells.

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Production of chimeric subviral particles with enhanced immunogenicity for the development of next-generation HBV vaccines

Norica Nichita [a]*, Ana-Maria Pantazica [a], Mihaela-Olivia Dobrica [a], Catalin Lazar [a], Catalin Tucureanu [b], Iuliana Caras [b], Irina Ionescu [b], Adriana Costache [b], Adrian Onu [b], Jihong Liu- Clarke [c], Crina Stavaru [b]

a) Institute of Biochemistry of the Romanian Academy, Bucharest, Romania
b) Cantacuzino" Medico-Military National Research Institute, Bucharest, Romania
c) NIBIO - Norwegian Institute for Bioeconomy Research, Ås, Norway
**email: norica70@yahoo.co.uk*

Introduction:

Chronic infection with Hepatitis B virus (HBV) is a major health issue world-wide accounting for more than 800.000 deaths annually, while a curable treatment is still missing. The development of HBV vaccines based on expression of the small (S) envelope protein in yeast has significantly reduced the morbidity and mortality associated with this disease. However, up to 10% of vaccinated adults fail to develop a protective immune response and virus mutations resistant to current vaccines continue to emerge, prompting further research into developing more immunogenic HBV antigens.

The large (L) envelope protein plays a critical role in HBV attachment to hepatocytes and is the target for virus neutralizing antibodies, which makes it a promising immunogen for inclusion in novel vaccine formulations. However, efficient expression of the L protein in recombinant systems is very difficult to achieve due to a significant cellular toxicity induced by its retention in the endoplasmic reticulum.

In this work we took advantage of the S protein ability to self-assemble into highly immunogenic sub-viral particles (SVP's) and used this molecular scaffold to create chimeric antigens which contain relevant structural and functional epitopes of the L and S proteins.

Materials and methods:

By using molecular biology tools, the newly designed S/L chimeric HBV antigens were cloned in expression vectors and produced in mammalian cells. Their antigenic and immunogenic properties were characterised in molecular detail by ELISA, immunoprecipitation and western blot. The antigen candidate with the highest expression and secretion levels, which retained the ability to assemble in SVP, was selected for further studies *in vivo*.

Results:

Analysis of the immune response induced in mice vaccinated with S/L chimeric antigens demonstrated improved immunogenicity and superior activation of humoral and cellular immune responses when compared with the S protein. This combined activation resulted in production of neutralizing antibodies against both wild-type and vaccine-escape HBV variants.

Conclusions:

Our results validate the design of chimeric HBV antigens and promote the novel S/L protein as a promising strategy for immunization of poor-responders to current HBV vaccines.

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Novel HTS cell based assay for cis acting protease activity identifies Hepatitis C Virus NS2 cysteine protease inhibitors with antiviral activity

Juncu Andrei [a], Daniel Ion [a], Paula Forian [a], Gabi Chiritoiu [a], Teodeor Sulea [a] , Ana Borota [b], Luminita Crisan [b], Alina Bora [b], Sorin Avram [b] , Ovidiu Vlaicu [c], Dan Otelea [c], Ursula Bilitewski [d], Laurentiu Spiridon [a], Liliana Pacureanu [b], Costin-Ioan Popescu [a*]

a*) Institute of Biochemistry of the Romanian Academy, 296 Splaiul Independentei, Bucharest, Romania*
b*) Institute of Chemistry "Coriolan Dragulescu" of the Romanian Academy, 24 Mihai Viteazu, Timisoara, Romania*
c*) National Institute for Infectious Disease "Prof. Matei Bals", 1 Dr. Calistrat Grozovici, Bucharest, Romania*
d*) Helmholtz Centre for Infection Research, 7 Inhoffenstraße, Braunschweig, Germany*
**email: pop@biochim.ro; popescu80@hotmail.ro*

Introduction: Proteases represent major drug targets for various viruses. Cis acting proteases play important roles in different viral families life cycle. Due to the available assays most of the protease inhibitors are targeting trans-acting protease. Hepatitis C Virus (HCV) poses a significant global health challenge, infecting approximately 58 million individuals worldwide. One promising drug target is the HCV NS2 cysteine protease which is an exclusive autoprotease. Our understanding of the precleavage form of HCV NS2 is limited and no cell based HTS assay is available to identify potent inhibitors.

Materials and methods: Advanced bioinformatic techniques were used for compound library selection. A cell based assay was optimized using heterologous protein expression from plasmids obtained by basic molecular biology techniques. High-throughput screening was performed by robotic liquid handling and hit confirmation followed in HCV cell culture system. Mode of action was explored by new bioinformatics techniques which sample the conformational space of the precleavage form of HCV NS2 protease at high speed.

Results: Potential HCV NS2 inhibitors were prioritized "in silico" in a targeted cysteine inhibitors library. In parallel, we optimized and automatized for HTS screening a cell based assay for HCV NS2 cis-protease activity. The false positive hits were filtered by a counterscreen. A secondary screen performed in HCV cell cultured system identified several chemical scaffolds with IC50s in the low micromolar range to pursue. Series of structure – activity relationship (SAR), bicistronic replicons and rapid sampling of the NS2-NS3 protease precleavage form informed about the hit mode of action. Moreoevr, a novel insight in the dynamics of HCV NS2 precleavage form was achieved.

Conclusions: Herein, we present a new HTS assay for HCV NS2 protease which may be extrapolated to other cis- acting proteases and innovative bioinformatics methods to investigate the conformational space of the precleavage form of a cis-acting protease helping to design new compounds targeting this family of enzymes.

Multifunctional complexes nanolipid - microbubbles for a targeted delivery of antitumor drugs by ultrasonography-assisted procedures

Mihai Adrian Socaciu [a,c], **Dumitrita Rugina** [b,c],
Zorita Diaconeasa [b,c], **Madalina Nistor** [b,c], **Carmen Socaciu** [b,c*]

a) Faculty of Medicine, University of Medicine and Pharmacy "Iuliu Hatieganu"Cluj-Napoca Romania;
b) University of Agricultural Sciences and Veterinary medicine, Cluj-Napoca, Romania
c) BIODIATECH- Research Centre for Applied Biotechnology in Diagnosis and Molecular Therapy, Cluj-Napoca, Romania;
**email: csocaciudac@gmail.com*

Introduction: New exploratory directions in nanomedicine involves a multidisciplinary knowledge regarding functional nanostructures which incorporate anticancer agents, new solutions for a targeted delivery, including ultrasonography for improved theranostics. Innovative solutions were recently focused on multifunctional complexes which include antitumor molecules into nanolipid structures (liposomes, nanolipid complexes) which are attached to microbubbles, as optimal vehicles, and best contrast agents to deliver such molecules to tumor targets [1-3].

Materials and methods: Experimental data including different protocols applied to incorporate plant-derived anticancer molecules, betulinic acid (BA) and a standardized triterpenoid extract (TT) comparative to Doxorubicin (as a gold standard) in liposomes and lipid nanospheres (NLC) are presented, as well their physical and chemical linkage to microbubbles. These supramolecular structures were used for a targeted delivery to Walker256 tumor cells via sonoporation, either in vitro or in vivo. The characteristics of such complexes are described considering their size and composition by DLS size measurements, UV-Vis spectrometry and LC-MS, cytotoxicity on Walker tumor cells, as well their stability. Protocols for sonoporation experiments in vitro and in vivo will be also presented

Results: The review combines the updated information and experimental data obtained in our laboratory: adapted recipes and experimental protocols to optimize the critical ratios between the nanolipid/anticancer molecules, the incorporation rate of anticancer molecules inside nanolipids, the ratios between the nanolipid complexes and microbubbles, their stability in time, for an improved delivery and efficiency against tumor cells in vitro and in vivo.

Conclusions: Current opportunities and challenges are presented, related to the future developments for improved targeted drug delivery via ultrasonography, to be applied using such multifunctional complexes in clinical therapy, valorizing the innovative solutions offered by nanobiotechology.

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Medical devices for skin-wound healing; from bench to *in vivo* study

Anca Roseanu [a]*, **Madalina Icriverzi** [a], **Paula Ecaterina Florian** [a], **Livia Sima** [a], **Oana Gherasim** [b], **Valentina Gurmazescu** [b], **Gabriel Socol** [b], **Fabiola Ionita** [c], **Cristin Coman** [c]

a) Institute of Biochemistry of the Romanian Academy, Splaiul Independentei 296, 060031 Bucharest, Romania;
b) National Institute for Lasers, Plasma, and Radiation Physics, 409 Atomistilor Street, 077125 Magurele, Romania
c) 'Cantacuzino' National Medico-Military Institute for Research and Developmen, Gheorghe Ionescu – Sisesti 14A, Bucharest, Romania
**email:* roseanua@gmail.com

Introduction: The skin wound healing is among the most complex physiological process in the human body. It is governed by sequential yet overlapping phases, including homeostasis, inflammatory, angiogenesis, growth, re-epithelisation, and tissue remodelling. Any aberration in the process may lead to excessive wound healing or chronic wound, thus hindering the normal physical function of skin. While several therapies for wound healing are available, these are only moderately effective. From a clinical point of view, topical application is attractive for full-thickness wound, due to reducing adverse effects on other organs. A promising strategy for a more efficient action is to develop/design patches with the capability of promoting dermis regeneration and provide support for epithelial skin. In this context, we have proposed a new formulation based on chitosan and loaded with anti-inflammatory, antioxidant and antimicrobial compounds and evaluated the wound healing potential *in vitro* and *in vivo*.

Materials and methods: The effect of proposed patches on the viability of THP-1 differentiated macrophages was assessed by MTS colorimetric assay. The anti-inflammatory potential was investigated using an experimental model of inflammation, macrophages stimulated with bacterial endotoxins and the level of pro-inflammatory cytokines (IL-6, PGE2) determined by ELISA method. In vivo effect on wound healing process was investigated on rats for 14 days. As a control, Hartmann standardised silver and unloaded patches were used. The average wound area was measured every 48h. Samples of wounds/ scars were collected on the 7th and 14th postoperative days and evaluated histological and immunohistochemically (H&E, Masson's Trichrome, collagen, α-SMA, CD31).

Results: No cytotoxic effect on cells was found in the presence of the new patches. Moreover, the new formulation was able to reduce LPS-induced inflammation by suppressing pro-inflammatory cytokine production *in vitro*. The *in vivo* studies showed that on day 14, healing process was similar for the animal groups treated either with new or standard Hartmann patches, respectively. Haematological analysis of blood samples and histopathological examination of skin samples collected on day 14 revealed a reduced number of inflammatory cells and no bacterial colonization. An almost complete wound closure and skin normal architecture was observed. Topical application of coated patches on the wounds of rats increased collagen synthesis, stimulated re-epithelialisation, fibroblast proliferation, accelerating wound healing process in rats.

Conclusions: Collectively, our study provides evidence that the topical application of proposed patches can promote the cutaneous wound healing process and represents a promising candidate for skin tissue therapy.

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Plant-produced Hepatitis C Virus E2-derived glycoproteins for vaccine development

Mihaela-Olivia Dobrica[a,*], Andre van Eerde[b], Catalin Tucureanu[c], Adrian Onu[c], Lisa Paruch[b], Iuliana Caras[c], Ene Vlase[c], Hege Steen[b], Sissel Haugslien[b], Dominic Alonzi[d], Nicole Zitzmann[d], Ralph Bock[e], Costin-Ioan Popescu[a], Crina Stavaru[c], Jihong Liu Clarke[b*], Norica Nichita[a*]

a) Department of Viral Glycoproteins, Institute of Biochemistry of the Romanian Academy, Splaiul Independentei 296, Bucharest, Romania
b) NIBIO - Norwegian Institute for Bioeconomy Research, Høgskoleveien 8, 1433, Ås, Norway
c) Cantacuzino" Medico-Military National Research Institute, Bucharest, Romania
d) Institute of Glycobiology, Department of Biochemistry, University of Oxford, Oxford, UK
e) Max Planck Institute of Molecular Plant Physiology, Potsdam-Golm, Germany
**email: olivia@biochim.ro, mihaelaolivia.dobrica@yahoo.com*

Introduction: Infections with Hepatitis C Virus (HCV) represent a major global health burden with 58 million chronically infected people and 290,000 deaths/year caused by severe liver complications. An efficient antiviral therapy has been recently developed, however, the access to diagnosis and treatment is limited. In these circumstances, prevention by vaccination remains the most effective way to control HCV infections. Currently, there is no commercial vaccine against HCV infection. Our study aims to develop and characterize HCV antigens as vaccine candidates in a cost-effective manner. We previously published the production in premiere of an HCV antigen in plants as an alternative system, by expressing the E1E2 envelope heterodimer in lettuce leaves [1]. We describe here the expression and characterization of glyco-engineered antigens derived fromE2 protein, the major HCV vaccine candidate, in *Nicotiana benthamiana,* to investigate the impact of N-glycan composition on their immunogenic properties.

Materials and methods: E2 envelope protein was transiently expressed in *N. benthamiana* and in mammalian cells, as control, and purified by lectin affinity followed by size exclusion chromatography. N-glycans were released by glycosidases treatment and analysed by NP-HPLC. The purified E2 conformation was assessed by binding to either specific antibodies or the HCV receptor, CD81, and its immunogenicity was studied following vaccination of BALC/c mice. The neutralizing capacity of the E2-induced antibodies was verified using the HCV pseudoparticle (pp) system [2]. We further designed a histidine tagged E2-derived construct lacking the transmembrane domain and the hypervariable region I. The novel antigen was expressed in *N. benthamiana* wild-type (in presence/absence of KDEL signal) and CRISPR/Cas9-edited to lack β-1,2-xylosyltransferase and α-1,3-fucosyltransferase activities (FX-KO). Antigens were purified by Ni affinity chromatography and used for N-glycan profile analysis by LC/MS.

Results: Our data indicate similar expression of the E2 antigen in plant leaves and mammalian cells. The comparative HPLC glycan analysis shows a different extent in the N-glycan trimming between the two systems. Plant-produced HCV E2 acquired the native conformation required for CD81 binding and triggered specific IgM and IgG response in mice, with significant neutralizing activity against HCV1a pp [2]. The truncated E2 antigens presented an improved expression yield in plants (up to 200 µg/g FW) and displayed similar electrophoretic mobility irrespective of the plant type, indicating that the N-glycan pattern is not grossly altered by the host. The N-glycosylation profiles of E2-derived antigens in wild-type and FX-KO plants are currently being analysed by mass spectroscopy.

Conclusions: Our results promote plants as suitable host and biotechnological platforms for cost-efficient production of functional complex antigens for vaccine development.

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Identification of novel potentially active chondroitin/dermatan sulfate domains in human decorin by ion mobility mass spectrometry

Mirela Sarbu [a], Raluca Ica [a], Alina D. Zamfir [a,b*]

a) Department of Condensed Matter, National Institute for Research and Development in Electrochemistry and Condensed Matter, Timisoara, Romania;
b) Department of Technical and Natural Sciences, "Aurel Vlaicu" University of Arad, Arad, Romania;
**email: alina.zamfir@uav.ro*

Introduction: Decorin (DCN), an ubiquitous component of the extracellular matrix, is a proteoglycan consisting of a core protein linked to a single hybrid glycosaminoglycan chain in which chondroitin sulfate (CS) and dermatan sulfate (DS) domains are interspersed. Hence, DCN-CS/DS chain is characterized by sequences of different lengths, epimerization, sulfation patterns and binding functions [1]. This structural and functional diversity gave rise to development of approaches for the identification of the biologically active motifs. In this context, we report here on the introduction of ion mobility (IMS) MS and MS/MS for structural analysis of DCN-CS/DS and discovery of novel potentially active domains.

Materials and methods: The hybrid CS/DS chain, released by β-elimination from DCN prepared from conditioned media of cultured human skin fibroblasts, was depolymerization by chondroitin AC I lyase and fractionated by size-exclusion chromatography. The collected oligosaccharide fractions, purified and dissolved in methanol were infused by (-)nanoESI into a Synapt G2S mass spectrometer and subjected to IMS MS and CID MS/MS. The signal was acquired at 1.4 kV ESI and 15 V cone voltage. IMS wave velocity was set at 650 m/s and IMS wave height at 40 V. CID was performed using collision energies of 30 eV.

Results: The IMS MS separation and screening in the negative ion mode allowed: i) the discrimination of CS/DS isobaric species by their separation according to the mobilities of their corresponding ions and ii) the detection in the DCN-derived CS/DS domains of novel over- and undersulfated CS/DS sequences which, in view of their sulfation status are, more likely, biologically active. Moreover, by IMS CID MS/MS, the new potentially active domains were structurally characterized in details in terms of epimerization and sulfation pattern. Fragmentation analysis data indicated that even misregulations in the sulfate distribution along the CS/DS chain could be discovered; CS/DS domains, placed initially in the category of regularly sulfated sequences due to the number of sulfates that equals the number of disaccharide repeats, were found to correspond, in fact, to abnormally sulfated structures.

Conclusions: Considering these results, we believe that the concept of the present IMS MS-based approach addresses to a better extent the needs of glycosaminoglycomics for faster, more reliable and more sensitive methodologies on one side and a few of the many questions still open, related to the structure and sulfation status of biologically active CS/DS domains in human decorin.

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Transcriptomic profiling of halophilic archaeon *Haloferax alxandrinus* DSM 27206T grown under silver stress

Doriana Mădălina Buda [a,*], **Horia Leonard Banciu** [b, c]

a) Doctoral School of Integrative Biology, Faculty of Biology and Geology, Babeș-Bolyai University, Cluj-Napoca, Romania;
b) Department of Molecular Biology and Geology, Faculty of Biology and Geology, Babeș-Bolyai University, Cluj-Napoca, Romania;
c) Centre for Systems Biology, Biodiversity and Bioresources, Faculty of Biology and Geology, Babeș-Bolyai University, Cluj-Napoca, Romania;
**email: doriana.buda@gmail.com*

Introduction: Organisms living in high salt environments face multiple challenges including the incidental presence of high heavy metal levels [1]. The present study focuses on the mechanisms of silver tolerance in the halophilic archaeon *Haloferax (Hfx.) alexandrinus* DSM 27206^T through transcriptome analysis. Gaining insights into metal-cell interactions in halophilic prokaryotes can contribute to the development of effective strategies for mitigating metal contamination of saline systems [2].

Materials and methods: The physiological response of *Hfx. alexandrinus* DSM 27206^T to silver ions was assessed in saline (20% NaCl w/v) liquid cultures with 0.1, 0.25, and 0.5 mM $AgNO_3$. Scanning electron microscopy coupled with energy dispersive spectrometry (SEM-EDS) analysis was employed to reveal the produced silver nanoparticles. Genomic sequencing was performed on *Hfx. alexandrinus* cells, while RNA extraction was conducted for transcriptomic analysis. The RNA-Seq data was validated using reverse transcription quantitative PCR (RT-qPCR).

Results: Optical density (OD_{623}) measurements showed a slight delay in archaeal cells cultivated with increasing $AgNO_3$ concentrations compared to the control (no silver) cultures. SEM-EDS analysis revealed the presence of extracellular silver-containing nanoparticles in cells exposed to 0.5 mM $AgNO_3$. Gene set enrichment analysis (GSEA) indicated that genes associated with basic cellular metabolism, genetic and environmental information processing were significantly altered in silver-stressed cells. Metal transporters and metal-related genes were strongly induced in response to silver exposure, suggesting their involvement in silver detoxification. Moreover, multiple genes involved in oxidative stress management, basic metabolism, and cellular motility were also differentially expressed in response to elevated Ag^+ levels. The RT-qPCR results confirmed the reliability and consistency of RNA-Seq data in identifying candidate gene responsive to heavy metal exposure.

Conclusions: The present study employed RNA-Seq analysis to uncover a specific response to silver-stressed *Hfx alexandrinus* cells, involving differential gene expression across various cellular processes. The essential role of the CopA copper ATPase in surviving silver exposure was identified, providing novel insights into the cellular components and mechanisms underlying heavy-metal tolerance in halophilic archaea.

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Targeting tumor cells and ovarian tumor microenvironment using tissue transglutaminase inhibitors

Livia E. Sima [a,*], Monica Tudor [a], Cristian Munteanu [a#], Gabriela Chiritoiu [a#], Florin Jipa [b], Stefana Orobeti [a,b], Felix Sima [b], Stefana M. Petrescu [a], Gary E. Schiltz [c], Daniela Matei [c,d]
 # these authors contributed equally

a) Institute of Biochemistry of the Romanian Academy, 296 Splaiul Independentei, Bucharest, Romania; b) National Institute of Laser Plasma and Radiation Physics, 409 Atomistilor Street, Bucharest-Magurele, Romania; c) Northwestern University, 303 E Superior Street, Chicago, USA; d) Jesse Brown VA Medical Center, 820 S Damen Ave, Chicago, USA
**email: lsima@biochim.ro*

Introduction: Tissue transglutaminase (TG2) is a multifunctional protein that was found overexpressed in many solid tumors, including ovarian cancer. Extracellular TG2 forms a molecular complex with fibronectin (FN) and integrin 1 (I1) thereby modulating "outside-in" signaling controlling adhesion. Our proposed inhibitor MT4 interferes with ovarian cancer (OC) cell adhesion by binding to TG2 and inhibiting its interaction with FN [1]. TG2 is a non-toxic druggable target. However, treatment with TG2-FN small molecule inhibitors (SMIs) sensitizes cancer cells to chemotherapy. Here we present the results obtained during testing of new MT-4 analogues. Also, we recently pursued a new approach to co-target TG2 with inhibitors blocking signaling pathways involved in cellular adaptation to MT-4. Further, we characterized the effects of TG2 targeting in 3D co-cultures of OC cells and normal ovarian fibroblasts (NOF). Next, $CD8^+$ T cell killing activity was measured upon inhibiting different TG2 activities.

Materials and methods: We used proximity ligation assay (PLA) to test TG2-I1 disruption by MT-4 analogues and immunofluorescence microscopy for cell adhesion analysis. Flow cytometry was employed to characterize pFAK expression and cell cycle profile in WT and TG2KO OC cells treated with inhibitors. We developed new phosphoproteomics protocols on SILAC-labeled cells to identify key pathways differentially upregulated in cells attaching on FN in the presence of MT-4. Tumor microarrays were used for multiplex IHC (mIHC) using TSA labeling for assessing TG2 expression by multiple lineages in tumors. DiI/DiO labeled OC cells and NOFs were used for heterosphere formation. NOF secretome was assessed by mass spectrometry, upon activation in the presence of SMI-exposed OC cells. Mouse spleen isolated $CD8^+$ T cells were treated with TG2 inhibitors and used in T cell killing assays in live cell imaging conditions.

Results: Out of the 5 new analogues, compound #3002 produced the highest destabilization of TG2- I1 interaction. Consequently, OC cells showed decreased adhesion onto FN, an attenuated pFAK signaling and cell cycle arrest. Phosphoproteomic analyses revealed upregulation of members of cell adhesion and survival pathways, upon adhesion to FN in the presence of MT4. Pharmacological co-targeting of TG2 and identified signaling proteins decreases CFU count, while increasing apoptosis in OC cells and spheroids. CompuSyn analyses showed synergistic effects for several combinations of TG2-FN SMIs and signaling pathway inhibitors. Next, we detected TG2 in -SMA$^+$ stroma of OC tumors using mIHC. *In vitro*, MT-4 prevented OVCAR5-NOF heterospheroids formation, and increased CCL2 and CXCL12 secretion by NOFs. Also, MT-4 did not interfere with *in vitro* T cell killing activity, which together with the enhanced immune response observed in TG2KO mice [2] supports TG2 as potential therapeutic target in OC.

Conclusions: These results encourage the development of TG2 inhibitors for translational applications and underline the importance of this protein in OC metastatic process.

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Robosample: Harnessing the power of constrained molecular dynamics and Gibbs sampling to investigate macromolecules

Laurentiu Spiridon [a,*], Teodor Asvadur Şulea [a], Victor Gabriel Ungureanu [a], Eliza Cristina Martin [a], Andrei Jose Petrescu [a]

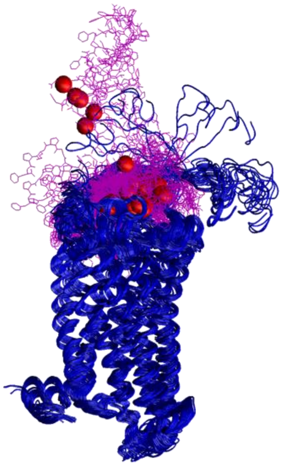
a) Institute of Biochemistry of the Romanian Academy 296 Splaiul Independentei, sector 6, Bucharest, Romania, 060031;
**email: spiridon.laurentiu@gmail.com*

Introduction: Simulation of macromolecules enables the investigation of the behavior, properties, and interactions of biomolecules. However, all the simulation techniques face a persistent obstacle posed by molecules' intricate potential energy landscape, arising from the interplay of high dimensionality and functional complexity. Within this work, we introduce Robosample [2], a software package that synergistically combines the efficiency of robot mechanics algorithms for rapid constrained simulations, with the ability of Gibbs sampling to jump across subspaces, making it capable to achieve statistical convergence hundreds of times faster.

Materials and methods: Robosample represents molecules as articulated rigid bodies, which offers a notable advantage in expediting macromolecule simulations primarily attributed to the ease of solving for constraints. An illustrative example is observed in torsional dynamics, wherein the system is transformed into Bond-Angle-Torsion coordinates, and then disregards the motion of bonds and angles. The equations of motion are efficiently solved within linear time, using robot mechanics algorithms with reduced coordinates. This eliminates the need for overdetermination, coordinate transformation, and mass matrix tensor inversion that would typically require cubic time complexity. The employed sampling technique, known as Gibbs sampling coupled with Hamiltonian Monte Carlo (GCHMC) [1] , selectively maps random variables across molecular degrees of freedom. Within GCHMC, specific rigid molecule segments are chosen to be subsampled, while soft degrees of freedom, such as torsions are oversampled. At the end, all degrees of freedom are sampled, interleaving fully flexible Cartesian Monte Carlo proposals.

Results: The method was successfully employed across a diverse range of molecules, encompassing both small compounds and large macromolecules. The method's validity was demonstrated on small systems, including a model four-bead linear system and alanine dipeptide, accurately reproducing their free energy landscapes. Subsequently, the efficiency of the method was evaluated by subjecting it to rigorous testing with macrocycle-containing molecules and complex glycan structures. These challenging systems provided a robust assessment of the method's performance in handling larger and structurally diverse molecules. To assess the scalability of the method for larger biological systems, GCHMC was subsequently tested on various challenging targets, including the HCV pre-cleavage viral glycoprotein NS2-NS3, human apelin APJR GPCR receptor, human Receptor for Advanced Glycation Endproducts (RAGE), and other important molecular structures. Simulation efficiency was assessed through comparative RMSD analysis with state-of-the-art available molecular simulation programs.

Conclusions: These diverse and complex biological systems served as robust benchmarks to evaluate the method's ability to effectively handle and accurately capture the dynamics and interactions within such large-scale systems.



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Computational assisted investigation of two key protein families of the immune system

Petrescu Andrei-J.

Department of Bioinformatics and Structural Biochemistry, Institute of Biochemistry of the Romanian Academy, Splaiul Independentei 296, Bucharest 060031, Romania

Introduction: Over the past decade our group have actively investigated by intertwining experiment with Bioinformatics and Biocomputing the structure-function relations in two major protein families of the immune system: (a) NOD like receptors - the key intracellular constituents of innate immunity and (b) the advent and evolution of the RAG system - found at the core of adaptive immunity.

Materials and methods: In order to predict the structure of the main modules and components we have developed intricate remote homolgy, ab-initio and constrained modelling techniques given the low level or absence of sequence similarity. Model based predictions were then validated by crystallography, FRET, cryoEM or point mutation analysis in collabortation with well established experimental groups in the field. Machine learning and bioinformatic techniques were further used to identfy structural invariants and motifs; and to gain an overall structured view of these highly diverse protein classes.

Results: Model based predictions combined with experiment were key to undersanding the processes and functionig cycles of these proteins as well as the determinant factors driving their properties. The work also resulted in useful computational tools and bioinformatic resources at hand now for the scientific community involved in immune system research.

Conclusions: Results presented herein clearly demonstrate that intertwining experiment with euristic molecular modeling, simulation and bioinformatics is able to crack down highly complex problems in structural biology which are out of reach to the most recent automatic approaches even if these are based on state of the art artificial intelligence methods.

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Zeaxanthin oil-in-water nanoemulsions as delivery systems for food or nutraceuticals applications

Adela Pintea [a,*], **Patricia Muntean** [a], **Andrea Bunea** [a], **Francis V. Dulf** [a], **Monica Focşan** [b],
Cristina Coman[a], **Dumitriţa Rugină**[a]

a) University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Calea Mănăştur 3-5, 400372, Cluj-Napoca, Romania;
b) Nanobiophotonics and Laser Microspectroscopy Center, Interdisciplinary Research Institute in Bio-Nano-Sciences, Babes-Bolyai University, 42 Treboniu Laurean, 400271 Cluj-Napoca, Romania
**email: apintea@usamvcluj.ro*

Introduction: Zeaxanthin and lutein, also known as the macular carotenoids are important contributor to eye and brain health and function. Due to their lipophilic character, the overall bioaccessibility of carotenoids from food or nutraceuticals is low. The lipid components in food are essential for the absorption of carotenoids and can modulate their bioaccessibility, depending on the concentration, unsaturation degree and chain length [1, 2]. The aim of our work was to develop oil-in-water nanoemulsions containing zeaxanthin (Zea) and zeaxanthin dipalmitate (ZeaDP) and to examine their impact on the bioaccessibility and cellular uptake of carotenoids.

Materials and methods: Four vegetable oils (coconut MCT, olive, sunflower, linseed and palm oils) were characterized in terms of fatty acids and carotenoid composition by GC-MS. Zea and ZeaDP were isolated and purified from plant sources using chromatographic techniques. Zea and ZeaDP oil in water (O/W; 1:9 v/v) nanoemulsions doped with carotenoids (0.05 mg/ml in the final emulsion) were obtained by probe ultrasonic emulsification, in the presence of emulsifier at 1 % (Tween 20). Bioaccessibility of carotenoids was determined by the INFOGEST standardized in vitro digestion protocol [3]. Cellular uptake in Caco-2 and RPE cells was determined by C30-HPLC-PDA and visualized using a Raman microspectrometer (InVia, Renishaw). Raman spectra were recorded with a 532 nm laser at a spectral resolution of ~4 cm-1. All data were analyzed using the QUASAR software and principal component analysis (PCA) was used to identify multiple cellular components.

Results: Very good incorporation yield (up to 98 %) of carotenoids in nanoemulsions were obtained for all vegetable oils. The average hydrodynamic diameter ranged between 100-200 nm (PDI < 0.3), depending on the type of oil (chain length, unsaturation degree). Nanoemulsions with ZeaDP had a larger diameter than those with free Zea, regardless of the type of fat used. The bioaccessibility of free zeaxanthin was higher than that of ZeaDP, regardless of the oil used, and higher for nanoemulsions compared to non-emulsified oils doped with carotenoids. Nanoemulsions with Zea obtained with coconut oil and those with ZeaDP and olive oil showed the highest bioaccessibility, with 60 % and 42%, respectively, which is far superior to the bioaccessibility of Zea from known food matrices. Cellular uptake and secretion of Zea in intestinal and retinal cells was superior from nanoemulsions compared to solvent delivery (DMSO), and dependent on the type of emulsions. The highest values were obtained for nanoemulsions with unsaturated oils. The presence of Zea in the cytosol of RPE cells, near the nucleus, was qualitatively confirmed by Raman mapping, based on specific and intense signals.

Conclusions: Oil-in water nanoemulsions dopped with zeaxanthin shows better bioaccessibility and cellular uptake compared to non-emulsified oils supplemented with zeaxanthin. Both the esterification of zeaxanthin and the type of oil impact the bioaccessibility of pigments. Zea nanemulsions represent promising delivery systems for food or nutraceuticals applications.

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Crimson serenade: Anthocyanins orchestrating melanoma defense, revealing oxidative stress and mitochondrial membrane potential through the enchantment of fluorescence free molecules and encapsulated brilliance

Dumitrița Rugină [a, *], **Mădălina Nistor**[a], **Zorita Diaconeasa** [a], **Monica Focșan**[b], **Luiza Găină**[c], **Mihai Cenariu**[a], **Roxana Pop** [a], **Carmen Socaciu**[a], **Adela Pintea**[a]

a) Biochemistry Department, University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Calea Mănăştur 3-5, Cluj-Napoca, Romania;

b) Nanobiophotonics and Laser Microspectroscopy Center, Interdisciplinary Research Institute in Bio-Nano-Sciences, Babes-Bolyai University, 42 Treboniu Laurean, 400271

c) Babes-Bolyai University, Faculty of Chemistry and Chemical Engineering, Arany János Street 11, Cluj-Napoca, 400028, Romania

**email: dumitrita.rugina@usamvcluj.ro*

Introduction: Anthocyanins (AnTs) are natural pigments that orchestrate a captivating display of color during autumn's leaf senescence, enchanting us with the sight of vibrant forests. Acting as protectors of vegetal tissues exposed to direct light, these remarkable molecules hold aesthetic beauty and profound health benefits. Humans have been consuming AnTs for centuries, effortlessly ingesting due to their presence in roots, fruits, leaves, juices, and extracts, embracing their presence without any concerns. However, the full journey of AnTS within cells once they are internalized remains partially veiled, therefore understanding of the AnTs intracellular path once they are uptaken by the cells needs more attention.

Methods and Results: Specifically, we have selected fruit extracts with a distinct chemical pattern (containing glucosylated derivatives or glycosylated) were selected to be tested on normal and melanoma cell lines, proving no detrimental effects on healthy cells, but reducing proliferation, increasing oxidative stress biomarkers, and diminishing the mitochondrial membrane potential in melanoma cells. The path we tread throughout our research was not without challenges like the intricate detection and monitoring of anthocyanins within the cells. In this light, diphenylboric acid 2-aminoethyl (DPBA), a non-fluorescent reagent, was successfully used to form fluorescent complexes with AnTs (aglycon and the glycosylated compounds as extract formula). According to NMR (nuclear magnetic resonance) spectroscopy and HRMS (high-resolution mass spectrometry) analysis, it was proven that DPBA and cyanidin are forming a complex, cyanidin@DPBA. Notably, *in vitro* tests the non-toxic nature of cyanidin@DPBA complex on B16-F10 melanoma cells. To further illuminate, the sub-cellular visualization of all AnTs, fluorescence microscopy and flow cytometry were employed, yielding detectable signals of non-metabolized cyanidin and glycosylated cyanidin within melanoma cells. Unfortunately, anthocyanins are very sensitive to environmental factors such as pH, light, and oxygen, and consequently are easily degraded. To counteract this challenge, an architectural structure was crafted, having a spherical shape, 1080 nm diameter, and a solid groundwork of $CaCO_3$ (PAH), on which rhodamine B isothiocyanate fluorophore was firstly added, was developed to counteract the anthocyanin's susceptibility to environmental factors. AnTs were entrapped between polyelectrolyte layers of the microcapsule (loading efficiency 94.6%). Moreover, these microcapsules offer the opportunity to trace the internalization and trafficking of AnTs, granting us glimpses into their mysterious journey after 24 hours of treatment.

Conclusions: Anthocyanins may inhibit melanoma cell proliferation and increase the level of oxidative stress, with opposite effect on normal cells. The effective technique to imaging AnTs is required in drug delivery development and mechanisms related to their metabolism. Through the artistry of encapsulation technology, we unveil valuable insights into the intricate delivery of anthocyanins within melanoma cells, presenting a strategic pathway that holds promise for enhancing tumor therapies.

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The neurological effects of *Glaucosciadium cordifolium* (Boiss.) Burtt & Davis essential oil in a zebrafish model of cognitive impairment

Razvan Stefan Boiangiu [a,*], **Eyup Bagci** [b], **Gabriela Dumitru** [a], **Lucian Hritcu** [a], **Elena Todirascu-Ciornea** [a]

a) Department of Biology, Alexandru Ioan Cuza University of Iasi, 700506, Iasi, Romania;
b) Department of Biology, Faculty of Science, Firat University, 23119, Elazig, Turkey
**email: razvan.boiangiu@uaic.ro*

Introduction: Alzheimer's disease (AD), the most common form of dementia, is characterized by progressive cognitive decline accompanied by mood changes, such as anxiety and depression. *Glaucosciadium cordifolium* has been used in traditional medicine for stomach ailments and as an aphrodisiac and appetizer and was proven to possess antimicrobial and antioxidant properties [1]. This study aims to investigate the *G. cordifolium* essential oil (GCEO) effects on anxiety and learning and memory impairment induced by scopolamine (SCOP) in zebrafish.

Materials and methods: The GCEO chemical composition was analyzed by GC-MS. The dementia model was induced by SCOP (100 µM), whereas GCEO (25 and 150 µL/L) and galantamine (GAL, 1 mg/L) were delivered to the SCOP-induced model. The anxiety-like behavior and cognitive performances of the animals were assessed using specific in vivo tasks, such as the novel tank diving test (NTT) and Y-maze and novel object recognition (NOR) tasks respectively. Post analysis, the animals were euthanized and the brains were used to measure the oxidative status and the acetylcholinesterase activity.

Results: The GC-MS analysis showed that the highest content was limonene followed by α- and β-pinene, p-cymene and α-phellandrene. It was found that GCEO significantly improved memory impairment and anxiety-like response induced by SCOP through the Y-maze, novel object recognition (NOR) test, and novel tank diving tests (NTT). Biochemical analyses showed that GCEO reduced SCOP-induced oxidative damage. Additionally, the cholinergic system activity was improved in the SCOP-induced model by decreasing the acetylcholinesterase (AChE) activity following the exposure to GCEO.

Conclusions: It was clear that as a mixture, GCEO displays positive action in improving memory impairment through restoring cholinergic dysfunction and brain antioxidant status.

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Young scientist's forum I

QbD based development of drug and genetic material co-loaded liposomes for regeneration of chondrocytes

Saketh Reddy Ranamalla [a,b], Alina Silvia Porfire [a], Emilia Licarete [c], Manuela Banciu [c], Ioan Tomuta [a]

a) Department of Pharmaceutical Technology and Bio Pharmacy, Faculty of Pharmacy, "Iuliu Hațieganu" University of Medicine and Pharmacy, 400010 Cluj-Napoca, Romania
b) Doctoral School in Integrative Biology, Faculty of Biology and Geology, "Babeș-Bolyai" University, 400015 Cluj-Napoca, Romania
c) Department of Molecular Biology and Biotechnology, Center of Systems Biology, Biodiversity and Bioresources, Faculty of Biology and Geology, "Babeș-Bolyai" University, 400015 Cluj-Napoca, Romania;
**email: saketh.ranamalla@ubbcluj.ro*

Introduction: Chronic knee and lower back pain due to osteoarthritis and intervertebral disc (IVD) degeneration have a global prevalence and impacts human wellbeing by impairing their mobility. Non-viral gene therapy offers a great advantage to restore the IVD and joints safely and precisely. This study is aimed at developing liposomal formulation for efficient co-delivery of curcumin and therapeutic siRNA. As curcumin downregulates many inflammatory cytokines along with free radicals and upregulates collagen and aggrecan, therefore reduces pain and helps in regeneration. In this study, luciferase siRNA is taken for screening and developing a successful prototype, that could transfect and internalise in luciferase expressing chondrocytes (C28/I2 cells) effectively.

Materials and methods: Preparation of liposomes with desirable physico-chemical and therapeutic properties is challenging as there are many variables and many responses interlinked. QbD approach was used for identification of critical factors, screening, and optimisation in a scientific manner. For our study, we have chosen particle size (PS), polydispersity index (PDI), zeta potential (ZP), encapsulation efficiency of curcumin (EE), cell viability, siRNA complexation capacity (at different Nitrogen: Phosphate [N/P] group ratios) and the luciferase activity to be the evaluated responses. After initial trials, Failure modes effects analysis (FMEA) was performed where the identified critical factors were the building components of the liposomes i.e., concentrations of the cationic (DOTAP) and helper lipids (DOPE/ DOPC/ DPPC), pegylated phospholipids (MPEG-2000-DSPE) and cholesterol. The total lipid content of the liposomes was kept constant at 20 mM. The impact of critical factors on the responses was studied through a Taguchi Orthogonal array design, developed using MODDE 13 statistical software by Sartorius. Cell internalisation was also determined with fluorescence and confocal microscopy. The responses obtained were entered back into the software to establish the relationship between the factors and to get an optimum formulation with highest cell transfection.

Results and discussion: An optimum formulation, with DOTAP, DOPE/ DPPC, Cholesterol, MPEG was established by the software. The liposomes were characterised to have desired PS, PDI, ZP and have given the highest knockdown, when tested in chondrocyte cell line.

Conclusion: These optimum formulations with curcumin and siRNA effectively transfect chondrocyte cells with no toxicity and could be successfully carried forward for testing in vitro in OA and IVDD models.

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Phenolic profile of micro- and nano-encapsulated olive leaf extract in biscuits during in vitro gastrointestinal digestion

Călina Ciont [a†], Graziana Difonzo [b], Antonella Pasqualone [b], Simona Maria Chis [a†],
Florica Ranga [a], Katalin Szabo [a], Elemer Simon [a], Anca Naghiu [c], Lucian Barbu-Tudoran [c],
Dan Cristian Vodnar [a], Oana Lelia Pop [a]*

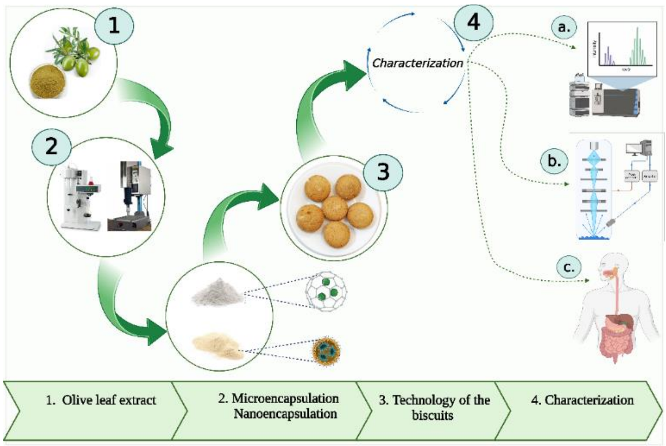
a) Department of Food Science, University of Agricultural Sciences and Veterinary Medicine,
400372, Cluj-Napoca, Romania
b) Department of Soil, Plant and Food Science (DISSPA), University of Bari Aldo Moro, I-70126 Bari, Italy;
[3] Research Institute for Analytical Instrumentation, National Institute of Research and
c) Development for Optoelectronics INOE 2000, 67 Donath Street, 400293 Cluj-Napoca, Romania;
**email: oana.pop@usamvcluj.ro*

Introduction: Olive leaf was characterized by a high content of phenols and flavonoids (oleuropein, luteolin, and their derivatives), presenting functional and health-related properties [1]. The chemical instability of phenolics through technological processes and their bioavailability may negatively impact them, leading to lower absorption [1,2]. This research aims to appraise the evolution of the phenolic extract from olive leaves and assess their stability and bioaccessibility through incorporation in biscuits matrix, using in vitro digestion model.

Materials and methods: The extraction process of olive leaves was ultrasound-assisted, and a binary blend of gum arabic and maltodextrin (1:1 w/w) was used for its encapsulation, confirmed through TEM and SEM microscopy.

Results: Applying the micro- and nano-capsules in biscuits showed significant protection of phenolic compounds. According to the in vitro results, the biscuits enriched with olive leaf extract can be considered a proposal for functional food products.

Conclusions: The present study has the opportunity to contribute significantly to the vast field of functional foods. It provides new options for producing healthier foods while optimizing the valorization of olive by-products.



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Hepatitis C Virus Envelope Protein E2 Antigen Design and Characterization for Vaccine Development

Lia-Maria Cucoș [a], Teodor Asvadur Șulea [a], Laurențiu Spiridon[a], Iuliana Caras [b], Irina Ionescu [b], Adriana Costache [b], Crina Stavaru [b], Norica Nichita [a], Costin-Ioan Popescu [a]

a) Institute of Biochemistry of the Romanian Academy, Bucharest, Romania
b) "Cantacuzino" Medico-Military National Research Institute, Bucharest, Romania
**email: lia@biochim.ro; liamcucos@gmail.com*

Introduction: With over 58 million individuals infected with Hepatitis C Virus (HCV) worldwide and costly antiviral drugs, the need for a vaccine is even more pressuring. The main target in vaccine development is the viral envelope protein E2, which binds to CD81 receptor functioning as an entry factor for HCV. HCV E2 412-423 epitope is involved in CD81 binding and it is targeted by broadly neutralizing antibodies (nAbs). HCV E2 412-423 epitope presents in different conformations due to protein flexibility and a beta hairpin conformation was found to co-crystalize with a nAb. The objective of our study was to construct and characterize novel HCV E2 derived antigens that elicit a potent neutralizing humoral response by stabilizing the 412-423 epitope in a beta hairpin conformation.

Materials and methods: Advanced bioinformatics tools were used to predict mutations stabilizing this epitope. Briefly, starting from a homology model built using Modeller, a conformational ensemble was generated using Hamiltonian Monte Carlo coupled with Gibbs Sampling (GCHMC), implemented in Robosample and followed by implicit solvent molecular dynamics (using OpenMM) to test mutant stability. A subset of HCV E2 mutants were expressed in HEK 293T cells and their biochemical and antigenical properties were investigated by Western Blot, ELISA, and glycan digestion. A vaccine candidate was expressed in Expi293 cells and the monomeric form was purified by IMAC (Immobilized metal affinity chromatography) followed by size exlusion chromatography to over 95% purity. The candidate was used to immunize BALB-C mice and obtained sera was used to neutralize HCVpp infection.

Results: Screening results provided us with an antigen candidate showing decreased binding for an antibody recognizing the open structure of the epitope. This antigen was successfully purified in the monomeric form. A mass spectrometry assay was optimized for further structure confirmation. The antigen was not able to bind CD81. Preliminary immunization studies revealed similar immunogenicity of the new antigen comparing to HCV E2 protein. Moreover, a potentially higher neutralization capacity of the immune sera was observed for the conformationally stabilized antigen using the HCV pseudotype system.

Conclusions: The presented data suggest that by de-novo antigen design, HCV E2 412-423 epitope can be stabilized in a desired conformation with potential impact on its immunogenicity.

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Reacting to stress: upregulating the genes involved in polyhydroxybutyrate synthesis during nutrient starvation by Halomonas elongata DSM 2581T

Andreea-Melisa Tripon [a,*], **Adorján Cristea** [b], **Horia Leonard Banciu** [c]

a) Doctoral School of Integrative Biology, Faculty of Biology and Geology, Babeș-Bolyai University, Cluj-Napoca, Romania;
b) Department of Taxonomy and Ecology, Faculty of Biology and Geology, Babeș-Bolyai University, Cluj-Napoca, Romania;
c) Department of Molecular Biology and Biotechnology, Faculty of Biology and Geology, Babeș-Bolyai University, Cluj-Napoca, Romania
**email: andreea.tripon@ubbcluj.ro*

Introduction: Nutrient starvation in some prokaryotes triggers a series of molecular events that culminate in the activation of polyhydroxyalkanoate synthase (PhaC), which is responsible for the synthesis of polyhydroxyalkanoates (PHAs). Among the PHAs, polyhydroxybutyrate (PHB) is the most abundant polyester. PHB serves as intracellular carbon and energy reserve in the extremely halotolerant bacterium *Halomonas elongata* DSM 2581^T during nitrogen limitation and abundant carbon availability [1]. Despite its prospective relevance as bioplastics precursor, the molecular mechanisms underlying PHB synthesis in *H. elongata* is poorly understood. Therefore, the objective of our study is to investigate the molecular processes involved in the synthesis and depolymerization of PHB in *H. elongata* DSM 2581^T.

Materials and methods: *H. elongata* was cultivated in rich and nutrient-limited saline media (8% NaCl, w/v) followed by biomass collection at 24, 96, and 120 hrs. Total RNA extraction was performed using the RNeasy PowerSoil Total RNA kit (Qiagen). The extracted RNA was prepared for RNA-Seq at Macrogen. The raw sequences underwent quality checking with FastQC v0.11.7, followed by trimming using Trimmomatic 0.38. Short RNA reads were mapped to the reference genome using Bowtie 1.1.2 and gene expression levels from the RNA-seq data were assessed using HTSeq version 0.10.0. The accumulation of PHB during the experiments was monitored using the spectrophotometric-based crotonic acid assay.

Results: PHB synthesis in *H. elongata* was induced under nutrient-limiting conditions, with the number of upregulated genes increasing over time as follows: 583 genes (24 hrs), 660 genes (96 hrs), and 716 genes (120 hrs). PHB production is relatively low (0.2 g/L ± 0.02) at 24 hours. Nevertheless, the expression of *phaC* and other genes associated with sugar transport and glucose metabolism, is increased compared to the control. In contrast, expression of *phaA* (coding for acetyl-CoA C-transferase) is downregulated. At 96 hrs, a significant increase in PHB is observed (1.8 g/L ± 0.06). *phaC* and genes related to sugar transport remain upregulated, while genes associated with glucose metabolism are downregulated. At 120 hrs, when PHB production plateaus (2 g/L ± 0.05), *phaC* remains upregulated while genes associated with sugar transport and metabolism are downregulated.

Conclusions: Our results suggest a redirection of metabolic resources towards PHB production in *H. elongata* grown under nutrient limitation. Further investigations, such as metabolic reconstruction, are needed to fully comprehend the regulation of sugar metabolism and other pathways involved in PHB synthesis. Overall, our study enhances understanding of PHB synthesis in *H. elongata* and provides a basis for future optimization of PHB production in halophilic bacteria.

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Overcoming doxorubicin resistance in B16.F10 melanoma cells by targeting Hypoxia-Inducible Factor-1 alpha

Bogdan Dume [a]*, Alina Sesărman [b], Manuela Banciu [b], Emilia Licărete [b]

a) Doctoral School in Integrative Biology, Faculty of Biology and Geology, “Babes-Bolyai” University, Cluj-Napoca, Romania;
b) Department of Molecular Biology and Biotechnology, Center of Systems Biology, Biodiversity and Bioresources, Faculty of Biology and Geology, “Babes-Bolyai” University, Cluj-Napoca, Romania;
**email:* bogdan.dume@ubbcluj.ro

Introduction: Hypoxia plays a critical role in promoting cancer progression and conferring resistance to conventional therapies. Among various malignancies, melanoma stands out as a particularly challenging cancer to treat, owing to its inherent resistance to both tumour cell and microenvironment-mediated drug resistance mechanisms. Adaptation to hypoxia is primarily regulated by the activity of Hypoxia-Inducible Factor-1 (HIF-1). HIF-1 serves as a central mediator in the cellular response to hypoxia and acts as a key regulator of O_2 homeostasis, controlling the expression of numerous genes involved in various aspects of oncogenesis. In this study, we used an *in vitro* approach to investigate the effects of HIF-1 inhibition on sensitizing B16.F10 murine melanoma cells to the cytotoxic drug doxorubicin to which melanoma cells have intrinsic resistance.

Materials and methods: Subconfluent B16.F10 murine melanoma cells were reverse transfected with HIF-1α specific siRNA and incubated in hypoxia (1% O_2) for 24 hours. On the second day, the cells were treated with doxorubicin and subsequently incubated for an additional 24 hours. Gene knockdown was verified by quantitative RT-PCR. Then, cell proliferation, migration and invasion capacity were quantified. Protein levels of HIF-1α and other pro-survival proteins were examined by Western blot analysis. Intratumour angiogenesis related protein production was determined by protein microarray. Total RNA was extracted and sent for transcriptome sequencing. MDA, a lipid peroxidation marker, was measured using HPLC to evaluate cell oxidative stress.

Results: The specific siRNA significantly decreased both gene and protein expression levels of HIF-1α, leading to an increased sensitivity of the cells to doxorubicin. Combined therapy showed a greater reduction of cell proliferation, migration and invasion capacity than cells treated with doxorubicin alone. Also, concomitant administration of HIF-1α siRNA and doxorubicin promoted apoptosis, induced oxidative stress and showed a modest inhibitory effect on angiogenesis.

Conclusions: HIF-1α knockdown emerges as a promising future treatment strategy in melanoma as it has shown significant results in its ability to overcome B16.F10 murine melanoma cell resistance to doxorubicin.

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“Humanization” of the N-glycosylation pathway in Nicotiana benthamiana via CRISPR/Cas9 technology significantly improves the immunogenicity of HBV antigens and the virus- neutralizing antibody response in vaccinated mice

Ana-Maria Pantazică [a,*], André van Eerde [b], Mihaela-Olivia Dobrică [a], Iuliana Caras [c], Irina Ionescu [c], Adriana Costache [c], Cătălin Tucureanu [c], Hege Steen [b], Cătălin Lazăr [a], Inger Heldal [b], Sissel Haugslien [b], Adrian Onu [c], Crina Stăvaru [c], Jihong Liu Clarke [b], Norica Nichita [a]

a) Institute of Biochemistry of the Romanian Academy, Splaiul Independentei no. 296, Bucharest, Romania
b) NIBIO - Norwegian Institute of Bioeconomy Research, P.O. Box 115, N-1431, Ås, Norway
c)”Cantacuzino” Medico-Military National Research Institute, Splaiul Independentei no. 103, Bucharest, Romania
**email: pantazicaanamaria@gmail.com*

Introduction: Hepatitis B Virus (HBV) infection affects over 290 million people worldwide, with more than 800 000 annual deaths due to HBV-related complications. Vaccination remains the best approach to mitigate HBV infections and meet the World Health Organization goal to eliminate viral hepatitis by 2030. Development of more immunogenic HBV antigens is crucial to overcome the disadvantages of the current vaccine, based on the small (S) envelope protein produced in yeast, such as non-responsiveness and lack of protection against “vaccine-escape” mutants. We have previously shown that combination of relevant immunogenic epitopes of the S and large (L) envelope proteins significantly increased the anti-HBV immune response. However, protein production in mammalian cells is costly and limits large scale production. Plants are an attractive alternative for protein production, as they are versatile and rapidly scalable. The recent generation of plants lacking β-1,2-xylosyltransferase and α-1,3- fucosyltransferase activities (FX-KO), by CRISPR/Cas9 genome editing, enables production of proteins with “humanized” N-glycosylation. In this study, we investigated the impact of plant N-glycosylation on the immunogenic properties of a chimeric HBV S/L vaccine candidate produced in wild-type and FX-KO *Nicotiana benthamiana*.

Materials and methods: Wild-type and FX-KO *N. benthamiana* plants were transformed by Agrobacterium infiltration with a vector encoding the chimeric S/preS1 antigen, followed by antigen extraction, molecular characterisation and purification. The N-glycosylation patterns of the HBV antigens were determined via UPLC-FLD/MS and antigenicity was determined via ELISA using a panel of conformation dependent anti-S antibodies. The antigens were used for immunization in mice and the humoral and cellular immune response was quantified by ELISA and ELISpot. The neutralization capacity of the obtained anti-sera was tested against wild-type and “vaccine escape” HBV by using an *in vitro* infection system.

Results: We found that the absence of β-1,2-xylose and α-1,3-fucose from the HBV antigen obtained in FX-KO *N. benthamiana* significantly impacted its antigenicity when compared to the wild-type counterpart. Moreover, the “humanized” glycosylation pattern increased the immune response in mice, as compared with the wild-type plant-produced antigen. Notably, the antibodies triggered by the FX-KO-produced antigen neutralized more efficiently both wild-type HBV and a clinically relevant vaccine escape mutant.

Conclusions: Our study validates in premiere the glyco-engineered *Nicotiana benthamiana* as a substantially improved host for plant production of glycoprotein vaccines and illustrates the importance of antigen glycosylation for the immunogenic properties of vaccine candidates.

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Simvastatin-induced energy metabolic shift towards lipid metabolism in B16.F10 murine melanoma cells

Giorgiana G. Negrea [a*], **Loredana Bălăcescu** [b], **Alina Sesărman** [c], **Manuela Banciu** [c]

a) Doctoral School in Integrative Biology, Faculty of Biology and Geology, "Babeș-Bolyai" University, Cluj-Napoca, Romania
b) Department of Functional Genomics, Proteomics and Experimental Pathology, Institute of Oncology "Prof. Dr. Ion Chiricuță" Cluj-Napoca, Romania
c) Department of Molecular Biology and Biotechnology, Center of Systems Biology, Biodiversity and Bioresources, "Babeș-Bolyai" University, Cluj-Napoca, Romania
**email:* giorgiana.negrea@ubbcluj.ro

Introduction: Malignant cells rely on enhanced glycolysis for ATP production, while normal cells primarily utilize oxidative phosphorylation. Simvastatin (SIM), a competitive inhibitor of 3-hydroxy-3-methylglutaryl-Coenzyme A reductase (HMG-CR), has demonstrated antitumoral effects at high doses [1]. This study investigated the effects of SIM on B16.F10 murine melanoma cells, focusing on energy metabolism. In tight connection with the primary pharmacological role of SIM on the inhibition of cholesterol synthesis pathway, we also explored statin impact on the transcriptional/post-synthetic regulation of this metabolic pathway, more specifically with regard to the sterol regulatory element binding transcription factors (SREBPs), and their interactions with SREBP cleavage-activating protein (SCAP) and insulin-induced gene protein (INSIG).

Materials and methods: RNA-seq analysis was conducted for mRNA expression, while western blot analysis assessed levels of glucose transporter 1 (GLUT1) and fatty acid synthase (FASN), and enzymatic assays measured catalytic activities of phosphofructokinase 1 (PFK1) and lactate dehydrogenase (LDH).

Results: SIM treatment significantly reduced glycolysis by decreasing the catalytic activities of PFK1 and LDH. However, this decrease in glycolytic flux led to a shift toward fatty acid metabolism, evidenced by upregulated FASN at mRNA and protein levels. Additionally, SIM treatment stimulated the expression of acyl-Coenzyme A oxidase (Acox3), a key enzyme in peroxisomal β-oxidation (FAO), compared to the control levels. Notably, our study showed upregulation of INSIG-1 and INSIG-2, while SCAP remained unchanged after SIM administration. Moreover, SIM treatment significantly reduced gene expression of SREBP1 and forkhead box M1 (FOXM1), a factor associated with mevalonate pathway regulation, tumorigenesis, and glucose metabolism. Nevertheless, several mevalonate pathway enzymes were upregulated after SIM administration, except for HMG-CR.

Conclusions: In summary, SIM effectively suppressed glycolysis in B16.F10 murine melanoma cells. Therefore, compensatory responses of the statin on *de novo* fatty acid synthesis and peroxisomal FAO were noted. Moreover, regulatory factors for cholesterol biosynthesis were affected by SIM administration. Notably, these transcription factors such as SREBPs orchestrate critical cancer processes *i.e*. cell proliferation and metastasis [2], while FOXM1 has been associated with epithelial-to-mesenchymal transition (EMT), drug resistance, and angiogenesis [3].

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Anti-dementia effects of mansorin A, mansonone G, and 6-paradol in zebrafish (*Danio rerio*)

Honceriu Iasmina [a,*], **Brinza Ion** [a], **Boiangiu Razvan Stefan** [a], **Hritcu Lucian** [a]

a) "Alexandru Ioan Cuza" University of Iasi, Department of Biology, Carol I Boulevard, No. 11, Iasi, Romania
**email: iasmina.honceriu@student.uaic.ro*

Introduction: Alzheimer's disease (AD) is the most common form of dementia. The patients manifest memory and cognitive impairments accompanied by depression and anxiety. The main pathologies are β-amyloid plaque accumulation, neurofibrillary tangles, and the death of cholinergic neurons, followed by neuroinflammation and increased oxidative stress. AD is fatal yet incurable and a priority in research. Natural bioactive compounds show promising anti-AD potential. That may be the case for mansorin A and mansonone G from *Mansonia gagei* and 6-paradol from *Zingiber officinale*. This study aims to determine whether mansorin A, mansonone G, and 6-paradol have anti-dementia effects.

Materials and methods: Mansorin A, mansonone G, and 6-paradol were chronically administered to adult zebrafish (*Danio rerio*) by immersion. Anxiety-like behavior was assessed using specific *in vivo* tasks: the Novel Tank Diving test (NTT) and Novel Object Approach (NOA) test, while memory performances were determined using the Novel Object Recognition (NOR) and Y-maze tasks. The specific activities of the antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase along with carbonylated proteins and malondialdehyde levels were also determined. The cholinergic status was evaluated by acetylcholinesterase activity.

Results: The used bioactive compounds improve spatial and recognition memory in zebrafish as indicated by NOR and Y-maze tasks and reduce anxiety-like behavior in NTT and NOA tests. They also improve the antioxidant status in zebrafish brain, as indicated by biochemical analysis.

Conclusions: Mansorin A, mansonone G, and 6-paradol exhibited promnesic and anxiolytic effects in zebrafish and show anti-dementia potential.

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Baicalein 5,6-dimethyl ether prevent scopolamine-induced memory impairment in zebrafish by increasing the creb1 protein level and the mRNA expression of bdnf and creb1 gene

Ion Brinza [a, *], Alexandru Bogdan Stache [a,b], Razvan Stefan Boiangiu [a], Omayma Eldahshan [c], Dragos Lucian Gorgan [a], Marius Mihasan [a], Lucian Hritcu [a]

a) Department of Biology, Alexandru Ioan Cuza University of Iasi, Iasi, Romania
b) Department of Molecular Genetics, Center for Fundamental Research and Experimental Development in Translation Medicine-TRANSCEND, Regional Institute of Oncology, 700483 Iasi, Romania
c) Department of Pharmacognosy, Ain Shams University, Egypt
**email: ion.brinza@student.uaic.ro*

Introduction: Baicalein 5,6-dimethyl ether (Baic), is a natural flavonoid, which was isolated for the first time from the roots of the plant *Scutellaria baicalensis*, variety, Georgi. Numerous studies have shown that Baic has many pharmacological properties and a variety of important biological activities such as its anti-inflammatory, anticancer and neuroprotective activity. However, its effects on the neuropathology of Alzheimer's disease (AD) have not been well studied [1,2]. AD is a neurodegenerative, multifactorial, progressive, and irreversible disorder, which involves various mechanisms of disease onset and progression. The disease is associated with memory disorders and cognitive decline that ultimately affect thought, reason, video-spatial orientation, and behavior. Neurovascular dysfunction, cholinergic changes, inflammatory processes, and oxidative stress are critical factors in the pathogenesis and development of the disease. Decreased autophagy and the ability to regulate the production of brain-derived neurotrophic factor (BDNF), nuclear factor erythroid 2-related factor 2 α (*nrf2α*) and cAMP response binding protein (CREB) are also reported as emerging disease factors. In the final stage of the disease, the affected people become unconscious and stiff.

Materials and methods: Baic (1, 3 and 5 µg / L) was administered by immersion to zebrafish once a day for 16 days. To assess the anxiety-like behavior of zebrafish, the novel tank diving test (NTT) was used. For assessing the spatial memory and the recognition memory, the Y maze test, and the novel object recognition test (NOR) were used. To evaluate the effects of Baic on the oxidative and cholinergic status in the zebrafishes brain, the specific activity of superoxide dismutase (SOD), catalase (CAT), malondialdehyde (MDA) level and acetylcholinesterase (AChE) activity were evaluated. Also, in this study the effects of Baic on *bdnf, nrf2α* and *creb1* mRNA expression were investigated and the level of CREB1 protein by the immunoblotting technique was quantified.

Results: Baic can improve cognitive dysfunction of the amnesic zebrafish by increasing the absolute gene expression of *bdnf, nrf2α* and *creb1*, and inhibiting AChE activity and restoring the antioxidant status, which is also correlated with improved memory parameters, as shown in behavioral approaches (NTT, Y-maze and NOR).

Conclusions: Our results suggest that Baic may be a novel active therapeutic drug for the amelioration of memory degradation.

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Young scientist's forum II

Advancing selenium analysis: method optimization, validation, and its application in assessing selenium levels in selenate-treated rats

Petruț Miron [a*], Bogdan Boșca [a], Andra-Diana Andreicuț [b], Alina Parvu [b], Augustin C. Moț [a]

[a]ANALYTICA Research Center, Faculty of Chemistry and Chemical Engineering, Babeș-Bolyai University, Cluj-Napoca, Romania.
[b]Department of Pathophysiology, "Iuliu Hațieganu" University of Medicine and Pharmacy, 400012 Cluj-Napoca, Romania
**email: petrut.miron@stud.ubbcluj.ro*

Introduction: Selenium is an essential trace element with diverse environmental forms, and it plays a vital role in biological systems. Inorganic selenium compounds, including selenite and selenate, facilitate its incorporation into the food chain, while organic selenium compounds, such as selenomethionine and selenocysteine, contribute to essential protein functions. The fluorescence properties of certain selenium compounds have attracted attention in bioimaging and biomedical research, enabling precise detection and real-time monitoring of molecules and structures in living systems. In our study, we introduce an optimized method to quantify selenium absorption in rat organs, providing insights into selenium metabolism and its health implications.

Materials and Methods: Targeted organs (heart, liver, and kidney) were mineralized in concentrated nitric acid and hydrogen peroxide. Selenate was chemically reduced to selenite, and the resulting selenite quantity was determined by measuring the fluorescence of piazselenol compound produced after the reaction with 2,3-diaminonaphthalene at 520nm. Measurements were performed using a fluorescence spectrometer, and a calibration curve was established using known selenite standards. The study followed statistical analysis, included replicates and controls, and adhered to ethical guidelines.

Results: The absorption of selenite demonstrated a proportional relationship with the quantity of ingested selenate across multiple experimental lots. The developed method allowed for clear signal detection without interference from other fluorescent species in the samples. This reliable methodology enables the quantification of selenite levels and investigation of the relationship between selenate ingestion and selenite assimilation in the examined organs.

Conclusions: Our findings highlight the influence of different selenium compounds, particularly selenate ingestion, on the overall assimilation of selenite in organs such as the kidney, heart, and liver. The optimized method provides a comprehensive understanding of selenium metabolism and opens avenues for further research on its impact on overall health and disease.

Keywords: selenium, fluorescence, quantification, piazselenol compounds.

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Evaluation of phenolic compounds from the morphological parts of beechnut (*Fagus sylvatica L.*)

Alexandra R. Lazăr [a,*], Andrei M. Mocan [b], Andreea Pușcaș [a], Andruța E. Mureșan [a], Floricuța Ranga [c], Alina M. Truță [d], Vlad Mureșan [a]

a) Food Engineering Department, Faculty of Food Science and Technology, University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, 3-5 Calea Mănăştur Street, 400372 Cluj-Napoca, Romania

b) Department of Pharmaceutical Botany, "Iuliu Hațieganu" University of Medicine and Pharmacy,Cluj-Napoca 400337, Romania

c) Department of Food Science, University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, 3-5 Mănăştur Str., 400372 Cluj-Napoca, Romania

d) Faculty of Horticulture, University of Agricultural Sciences and Veterinary Medicine of Cluj-Napoca, 3-5 Mănăștur St., 400372 Cluj-Napoca, Romania

**email: alexandra-raluca.lazar@student.usamvcluj.ro*

Introduction: The Plant Kingdom is an unlimited supply of natural bioactive substances that may be used in a variety of sectors, including food, medicine, and cosmetics [1]. The European beech (*Fagus sylvatica* L.) is one of the most significant tree species in the northern hemisphere, with a kernel that is a triangular, reddish-brown achene that is 1-1.5 cm long and encased in an involucre [2]. The current study attempted to determine the biologically active compounds found in the involucre, achene, skin, and cotyledon of the beechnut seeds.

Materials and methods: The raw materials were harvested from the production unit (U.P.III. Chelința, Maramureș), from the landscaping unit (u.a. 71 A) and subjected to the drying process at a temperature of approximately 15°C. About 1 g of each sample – the involucre, achene, skin, and cotyledon – was weighed and ground using a coffee grinder until a powder was obtained. Each sample was transferred into stoppered Erlenmeyer beakers and approximately 200 mL of methanol was added. The samples were then subjected to the sonication process (Bandelin SONOREX, Sigma Aldrich, Berlin, Germany) at a frequency of 80 Hz for 10 minutes. A concentration step was also performed at a temperature of 35°C under low pressure using a Heidolph Rotovapor. 10 ml of HPLC methanol were used for sample recovery, after which they were filtered through 0.45 µm Millipore nylon filters and used for HPLC-MS analysis. HPLC analysis was performed on Agilent 1200 system equipped with a quaternary pump, a degasser DGU-20 A3 (Prominence), an autosampler, an UV–VIS detector with a photodiode (DAD), coupled with a single-quadrupole Mass Detector Agilent model 6110 (Agilent Technologies, Santa Clara, CA, USA).

The phenolic compound separation was conducted on the Eclipse XDB C18 column (5 µm, 4.6 × 150 mm). The gradient program was adapted from (Weisz, Kammerer and Carle, 2009), as follows: 10% B to 17.2% B (18 min), 17.2% B to 23% B (12 min), 23% B isocratic (10 min), 23% B to 31.3% B (13 min), 31.3% B to 46% B (12 min), 46% B to 55% B (5 min), 55% B to 100% B (5 min), 100% B isocratic (8 min), 100% B to 10% B (2 min), 10% B isocratic (5 min). The total run time was 90 min. The injection volume for all samples was 5 lL. Phenolic compounds were monitored separately at 280 nm (hydroxybenzoic acids) and 320 nm (hydroxycinnamic acids) at a flow rate of 0.4 mL/min.

The chromatograms were monitored at 280 and 340 nm, respectively. The mass spectrometric data were obtained using a single-quadrupole 6110 mass spectrometer (Agilent Technologies, Chelmsford, MA, USA) equipped with an ESI probe. The measurements were performed in the positive mode with an ion-spray capillary voltage of 3000 V and a temperature of 300°C. The nitrogen flow rate was 7 L/min. Data were collected in full scan mode within 100 to 1200 m/z. The data reading and acquisition were made using Agilent ChemStation software.

Results: Results revealed 2065.26 mg / 100 g dw phenolic compounds in the involucre, among them, epicatechin (231.84 mg / 100 g dw) and procyanidin trimer isomer 1 (231.08 mg / 100 g dw) being more prevalent, followed by procyanidin trimer isomer 2 (228.45 mg / 100 g dw) and procyanidin trimer isomer 3 (226.90 mg / 100 g dw). Other compounds found in considerable amounts are catechin (216.66 mg / 100 g dw) and ellagic acid-arabinoside (213.17 mg / 100 g dw). Quercetin, kaempferol, kaempferol-di p-coumaroyl-glucoside, tiliroside, hydroxybenzoic acid, and procyanidin dimer isomer 2 are other compounds identified in the involucre. Eight phenolic compounds were identified in the achene - epicatechins, catechins, isomer 2 of the dimer and trimer of procyanidin, hydroxybenzoic acid, and in much smaller quantities naringenin-glucoside, tiliroside and kaempferol. Hydroxybenzoic acid was predominant in the beechnut skin (297.88 mg / 100 g dw), while in the cotyledon it was 54.18 mg / 100 g dw. The most significant proportion was provided by kaempferol-p-coumaroyl-glucoside (85.76 mg / 100 g dw), hydroxybenzoic acid-glucoside (83.77 mg / 100 g dw), and kaempferol-rutinoside (73.21 mg / 100 g dw) from the overall concentration of phenolic compounds in the cotyledon (627.07 mg / 100 g dw).

Conclusions: The four samples contained 24 phenolic compounds, the majority of which belonged to the flavanol subclass. Hydroxybenzoic acid and tiliroside were among the compounds discovered in varying levels in all four samples, with the rest being part-specific compounds. Phenolic compounds identified in the morphological parts of the *Fagus sylvatica* L. might represent a potential source of natural antioxidants for the food industry.

Fig.1 The morphological parts of beechnut

1 – involucre,
2 – achene,
3 – skin, and
4 – cotyledon

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In-silico Study of RAGE-S100B Inhibitors

Victor Gabriel Ungureanu[*a], **Ioana Popa, Carmen Tănase, Laurențiu Spiridon**[a]

a) Institute of Biochemistry of the Romanian Academy, Splaiul Independentei 296, Bucharest, Romania;
**email: victorungu99@gmail.com*

Introduction: RAGE (Receptor for Advanced Glycation Endproducts) is a transmembrane receptor which comprises a V-type domain, two C-type Ig-like domains (C1 and C2), a transmembrane helix, and a cytosolic domain. It is known to bind a wide variety of ligands, including the S100 calcium-binding protein B (S100B). Based on predictions from native and cross-linking mass spectrometry, it is suggested that these proteins form a multimeric complex through concurrent interactions between RAGE-RAGE and RAGE-S100B. The inhibition of RAGE activation could be the key to preventing the progression of multiple diseases such as Alzheimer's, inflammatory complications of diabetes and tumor progression. In this study, molecular docking was employed to identify potential inhibitors of the RAGE-S100B complex formation, from a commercially available library of 137 natural and small-molecule compounds, hereafter referred to as the "target set".

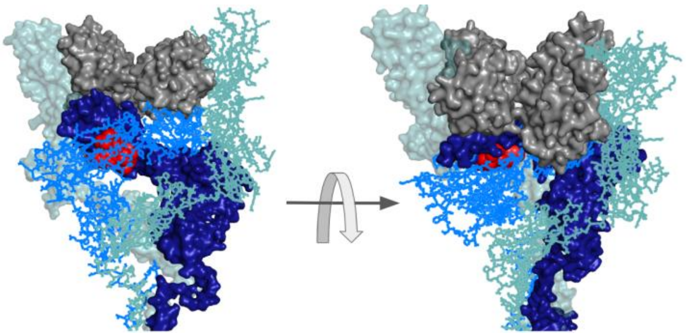
Materials and methods: RAGE and S100B receptor structural models were generated via homology modeling using Modeller. Protonation at the physiological pH of 7.4 and charge assignment were performed with *pdb2pqr* using the AMBER force field.

Prior to the docking and scoring of the target set, we created a "method selection set" by combining known BindingDB database RAGE binders with decoys generated through DeepCoy. Subsequently, the method selection set underwent molecular docking using multiple programs, including AutoDock4, Vina, Vinardo, DLigand2, GNINA, DrugScoreX and dkoes. We systematically evaluated each program and handpicked those that demonstrated superior discrimination between binders and decoys. We assume that an optimal score, by its very nature, would inherently indicate the correct pose. Following the docking of the method selection set, AutoDock was chosen for pose prediction, while dkoes was employed for ranking purposes.

Molecular docking search space was limited to four surfaces on VC1 that are known to be involved in the interaction between RAGE and S100B. The first surface consisted of amino acids already established to be involved in S100B binding, while the other three surfaces were suggested by previous studies [3] as key binding sites for different inhibitors.

Results: Our predictions indicate that the target set encompasses a range of mechanisms of action. Specifically, certain compounds bind to the RAGE-S100B interface, while others interact with the RAGE-RAGE interface. This observation suggests that the prevention of receptor oligomerization and inhibition of RAGE-S100B interaction are distinct mechanisms employed by different compounds to exert their inhibitory effects. Understanding these distinct mechanisms is crucial for comprehending the inhibitory action of specific compounds on cellular RAGE. The results of the in silico screen were compared with the in vitro binding results with purified proteins and cellular RAGE.

Conclusions: In this study, we have undertaken the optimization of a method specifically designed for the in silico detection and characterization of inhibitors that target the RAGE receptor. This optimized method serves as a valuable tool for virtual screening of larger compound libraries, enabling the identification of potential therapeutic candidates for RAGE-associated disorders.



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Preliminary results on the recovery of coenzyme Q10 from vegetable and animal waste

Andersina Simina Podar [a,*], **Cristina Anamaria Semeniuc** [a], **Floricuța Ranga** [a], **Maria-Ioana Socaciu** [a],
Melinda Fogarasi [a], **Anca Corina Fărcaş** [a], **Dan Cristian Vodnar** [a], **Simona Raluca Ionescu** [a],
Sonia Ancuța Socaci [a]

a) Faculty of Food Science and Technology, University of Agricultural Sciences and Veterinary Medicine
from Cluj-Napoca, Calea Mănăștur 3-5, 400372 Cluj-Napoca;
**email: andersina-simina.podar@usamvcluj.ro*

Introduction: Most foods of animal origin, such as meat, eggs and dairy products, are rich sources of coenzyme Q10 (CoQ10); other available food sources include vegetable oil, fish, bee pollen and microorganisms. This work aims to recover CoQ10 from plant and animal waste, in order to use it as a food supplement [1, 2].

Materials and methods: Six types of scraps, resulting from the cold extraction process of rapeseed, sunflower, pumpkin, linseed, walnut and hemp oils, minced samples of whole fish and chicken hearts, were studied to determine the content of CoQ10. CoQ10 extraction was performed by ultrasound-assisted extraction using 2-propanol as solvent.

Results: Among the studied plant extracts, pumpkin extract has the highest CoQ10 content (84.80 µg CoQ10/g material), and among the animal samples, the highest amount was determined in chicken hearts (114.39 µg CoQ10/g material).

Conclusions: Ultrasound-assisted extraction using 2-propanol as solvent is an effective method for the recovery of CoQ10 from both plant and animal matrix; the optimized CoQ10 recovery method has the advantage of being a simple and ecological method.

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Unraveling molecular basis of myeloproliferative neoplasms (MPN) by experimentally constrained modelling and simulation

Şulea A. Teodor [a,*], Nicolas Papadopoulos [b], Stefan N. Constantinescu [b], Laurentiu Spiridon [a], Petrescu J. Andrei [a]

a) Department of Bioinformatics and Structural Biochemistry, Institute of Biochemistry of the Romanian Academy, Splaiul Independentei 296, Bucharest 060031, Romania
b) Université catholique de Louvain and de Duve Institute, Brussels, Belgium
**email: teodor.sulea@biochim.ro*

Introduction: CALR is an ER-resident chaperone involved in glycoprotein folding quality control. In its wildtype form, this transitory protein holds glycoproteins in ER until their proper folding. However myeloproliferative neoplasms (MPN) were shown to result from several frameshift mutations in CALR which then permanently binds the Thrombopoietin receptor (TpoR) and disrupts its proper function. We aimed here to model the interaction between TpoR and mutant CALR in an effort to uncover the mechanism behind this specificity.

Materials and methods: Interaction between the distal Cytokine Receptor Module (CRM) of TpoR and the frameshift region of CALR was investigated by docking followed by Molecular Dynamics (MD) based Molecular Mechanics Generalised Born & Surface Area Solvation (MM-GBSA) and Time-lagged Independent Component Analysis (TICA) to identify conformational microstates. The overall tetrameric system (2 CALR-2 TpoR) system was then built incorporating Hydrogen-Deuterium Exchange data. This glycoproteic model was further inserted into a POPC lipid bilayer, accommodating the transmembrane region of the TpoR using known NMR constraints resulting in an overall model of ~1,000,000 atoms including water & ions which was then subjected to stability test via MD simulation.

Results: Relying exclusively on an EpoR template, the automated Alphafold2 model of TpoR-CRM fails to identify its true 4/5 β-sandwich nature due to its tendency to overfit with the closest template EpoR which displays a 3/4 β-sandwich architecture leaving a ~60aa sequence stretch unassigned. This was corrected by manual heuristic modelling. Interaction studies demonstrate that the frameshift CALR region tightly binds TpoR in a large number of configurations. This protein-protein interaction adds up to the lectin type interactions between the globular CALR region with TpoR immature glycans attached to N117.

Conclusions: During folding the N117 TpoR glycan is recognized by CALR-del52 mutant by the lectin mechanism. This brings TpoR-CRM to come in contact with the positively charged frameshift region of CALR-del52 stabilizing the formation of robust 2xTpoR-2xmCALR tetramer which prevents TpoR from working properly. Hence this work suggests on how mutations in ~~an~~ CALR have pathological effects and lays the foundation for possible inhibitory therapies as identifying the specific residues of CRM1 involved in CALR-del52 interaction might help in the development of inhibitors targeting this interaction.

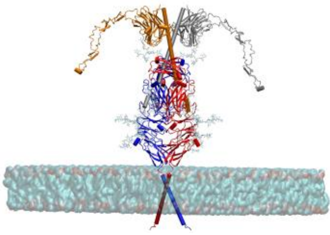

Fig 1 - The complete tetrameric model of the TpoR/CALR del52 model (Red/Blue Cartoon - TpoR; Orange/Grey Cartoon – CALR; Cyan Lines – Glycans; Transparent Surface – POPC Bilayer).

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Long-read direct RNA nanopore sequencing of the nicotine-related transcriptome of Paenarthrobacter nicotinovorans ATCC 49919

Amada El-Sabeh*, **Marius Mihășan**

Department of Biochemistry and Molecular Biology, Alexandru Ioan Cuza University, Iasi, Romania
**email: amadaelsabeh@gmail.com*

Introduction: *Paenarthrobacter nicotinovorans* ATCC 49919 is a soil nicotine-degrading bacterium that harbours the pAO1 catabolic megaplasmid. Although the nicotine catabolic genes have been sequenced, there is a gap in knowledge regarding the interplay between the degradation pathway and the general metabolism of the cell. A better understanding of the mechanisms which regulate nicotine catabolism would unlock the strain's potential in converting the toxic alkaloid into valuable precursors for drug synthesis and green chemicals. Here, long-read direct RNA nanopore sequencing was used to study the transcriptome of *P. nicotinovorans* ATCC 49919 grown in the presence and absence of nicotine.

Materials and methods: The bacterium was grown on citrate medium with and without nicotine. Cells were harvested at three timepoints correlated with nicotine catabolism (~5, 10 and 24 h after inoculation). Native RNA was extracted with the Promega SV Total RNA Isolation System. Because library preparation was performed with the Oxford Nanopore Technologies (ONT) Direct RNA sequencing kit (SQK-RNA002) designed for eukaryotes, adenine tails are required for ONT sequencing adapter ligation. Therefore, using the Lucigen Poly(A) Polymerase Tailing Kit, the extracted *P. nicotinovorans* native RNA was incubated with the *E. coli* poly(A) polymerase I to provide the necessary adenine tails for adapter ligation and sequencing. The applied library prep protocol and data acquisition parameters were adjusted for use with the ONT Flongle instead of the SpotON flow cell. Direct RNA sequencing was performed with the ONT MinION Mk1B device equipped with Flongle flow cells and controlled via the MinKNOW software. Raw data was basecalled with the Guppy_6.3.2 high accuracy algorithm. The resulting RNA sequences were quality controlled and analysed for differential gene expression between control and nicotine-treatment using the nf-core/nanoseq pipeline v3.1.0 [1].

Results: A total of 24 sequencing runs were performed, representing 4 replicates per timepoint and treatment, with a yield of 1 million reads totalling over 1 Gb, 80% of which had quality scores above 7. The longest read measured ~12 kb and the average read length was 1.2 kb. Differential expression (DE) analyses were performed with DESeq2. Over 40 genes with nicotine-related expression ($padj<0.1$; $abs(log2FoldChange)>1$) were identified: 15 were previously known to be involved in nicotine catabolism and the remaining genes are reported here first. Time-based analyses indicated the presence of nicotine-related transcripts up to ten hours after inoculation. 24 h after inoculation there was only one statistically significant ($padj<0.1$) DE transcript between control and nicotine-treatment, encoding for a universal stress protein previously linked to resistance to oxidative stress [2].

Conclusions: This study represents the first transcriptomic analysis of *P. nicotinovorans* ATCC 49919. Differential expression analyses identified over 20 novel genes which are suggested here first as having nicotine-related expression.

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POSTERS: Bio and nanotechnologies for biomedical and environmental applications

pH-responsive nanocapsules for anthocyanins encapsulation, delivery, intracellular tracking, and controlled release inside B16-F10 melanoma cells

Adela Maria Dăescu[1], Mădălina Nistor[1], Monica Focșan[2], Roxana Pop[1], Adela Pintea[1], Maria Suciu[3,4] and Dumitrița Rugină[1,*]

a) Faculty of Veterinary Medicine, University of Agricultural Sciences and Veterinary Medicine, 3–5 Mănăștur Street, 400372 Cluj-Napoca, Romania
b) Nanobiophotonics and Laser Microspectroscopy Centre, Interdisciplinary Research Institute on Bio-Nano-Sciences, Babes-Bolyai University, 42 Treboniu Laurean Street, 400271 Cluj-Napoca, Romania
c) Electron Microscopy Centre "C. Craciun", Biology and Geology Faculty, Babes-Bolyai University, 5-7 Clinicilor Street, 400006 Cluj-Napoca, Romania
d) National Institute for Research and Development of Isotopic and Molecular Technologies, 67-103 Donat Street, 400293 Cluj-Napoca, Romania
**email: dumitrita.rugina@usamvcluj.ro*

Introduction: Melanoma, the most lethal form of skin cancer, is characterized by the accumulation of a large load of mutations in melanocytes, mainly due to prolonged exposure to ultraviolet rays. Due to the multiple effects demonstrated by anthocyanins (AntS) in suppressing the expansion of melanoma, they can be considered promising sources of therapeutic molecules. The present article aimed to manufacture a fluorescent pH-sensitive polymeric nanosystem (Nano@AntS), capable of encapsulating the AntS extracted from *Aronia melanocarpa* fruits, that travels through the cellular environment and resists at a continuous decrement of pH, thus maintaining the AntS's stability.

Materials and methods: The extracted anthocyanins were purified using the solid phase extraction (SPE) technique and then subjected to HPLC chromatography. The purified anthocyanins were nano-encapsulated. The synthesis of Nano@AntS was done using a layer-by-layer technique, their morphology was examined by scanning and transmission electron microscopy (SEM/TEM) and the mean size was determined by DLS analysis. The presence and arrangement of fluorophores RBITC (561nm) and FTIC (468 nm) in the structure of the nanocapsules were verified using conventional fluorescence microscopy, confocal microscopy with double scanning RCM-VIS, and fluorescence microscopy (FLIM). WST-1 viability assay was used to determine the viability of the B16-F10 murine melanoma cell line after nanocapsules treatment. The FLIM and TEM microscopy techniques were used for the delivery, internalization, and intracellular localization of Nano@AntS in melanoma cells.

Results: Nano@AntS was successfully constructed by layer-by-layer technique with a final size between 50-200 nm and an encapsulation efficiency of AntS of 63%. The optimal release of AntS was obtained in solution at a pH value of 4.5 and 6.5. The system also induced maximal cytotoxicity of 40% on the tested cells, and the effect of AntS as a pure extract was highly cytotoxic in a dose-dependent manner. The localization and internalization of Nano@AntS were mostly observed in the cytoplasm.

Conclusions: This research successfully proposed and described a novel targeted drug delivery nanosystem with a modulated pH response, offering a potentially effective treatment for melanoma, adequate intracellular delivery of anthocyanins, and controlled response in the internal pH environment of melanocytes.

Acknowledgment: This research was funded by the Romanian National Authority for Scientific Research, CNDI– UEFISCDI, project number PN-III-P1-1.1-TE-2019-1276.

Design and preclinical testing of an anti-CD41 CAR T cell for the treatment of acute Megakaryoblastic leukemia

Adrian Bogdan Tigu [a*], Catalin Sorin Constantinescu [a,b,c], Patric Teodorescu [b,d], David Kegyes [a,b], Raluca Munteanu [a], Richard Feder [a], Mareike Peters [a,b], Ioana Pralea [a], Cristina Iuga [a], Diana Cenariu [a], Andra Marcu [e,f], Alina Tanase [e,f], Anca Colita [e,f], Rares Drula [a], Jon Thor Bergthorsson [g,h], Victor Greiff [i], Delia Dima [j], Cristina Selicean [j], Ioana Rus [j], Mihnea Zdrenghea [j], Diana Gulei [a], Gabriel Ghiaur [a,d], Ciprian Tomuleasa [a,b,j]

a) Medfuture Research Center for Advanced Medicine, Iuliu Hatieganu University of Medicine and Pharmacy, Cluj-Napoca, Romania;
b) Department of Hematology, Iuliu Hatieganu University of Medicine and Pharmacy, Cluj-Napoca, Romania;
c) Intensive Care Unit, Emergency Clinical Hospital, Cluj-Napoca, Romania;
d) Department of Leukemia, Sidney Kimmel Cancer Center at Johns Hopkins, Johns Hopkins University School of Medicine, Baltimore, MD, United States;
e) Department of Pediatrics, Carol Davila University of Medicine and Pharmacy, Bucharest, Romania;
f) Department of Stem Cell Transplantation, Fundeni Clinical Institute, Bucharest, Romania;
g) Stem Cell Research Unit, Biomedical Center, School of Health Sciences, University of Iceland, Reykjavík, Iceland;
h) Department of Laboratory Hematology, Landspitali, University Hospital, Reykjavík, Iceland;
i) Department of Immunology, University of Oslo and Oslo University Hospital, Oslo, Norway;
j) Department of Hematology, Ion Chiricuta Clinical Cancer Center, Cluj Napoca, Romania.
**email: adrianbogdantigu@gmail.com*

Introduction: Acute Megakaryoblastic leukemia (AMkL) is a rare subtype of acute myeloid leukemia (AML) representing 5% of all reported cases, and frequently diagnosed in children with Down syndrome. Patients diagnosed with AMkL have low overall survival and have poor outcome to treatment, thus novel therapies such as CAR T cell therapy could represent an alternative in treating AMkL. In the last decades, significant progress has been made in the field of novel immunotherapeutic agents that can be used as treatments for hematological malignancies. Chimeric antigen receptor T cells (CAR T cell) are the T cells modified to recognize specific antigen.

Currently, five FDA-approved CAR-T cell therapies are used in the clinic. This is the case for the antiCD19 CAR T cells tisagenlecleucel, brexucabtagene autoleucel, lisocabtagene maraleucel and axicabtagene ciloleucel, as well as the anti-BCMA idecabtagene vicleucel. Thus, the existing CAR T cells are mostly targeting CD19, which is a specific antigen for B cells, with impressive for B-cell acute lymphoblastic leukemia and B-cell non-Hodgkin lymphomas.

Materials and methods: The cell populations were evaluated by Flow cytometry, cytokine evaluation by ELISA Assays and LDH using a colorimetric test.

Results: The performed flow cytometry evaluation highlighted a percentage of 93.8% CAR T cells eGFP-positive and a limited acute effect on lowering the target cell population. However, the interaction between effector and target (E:T) cells, at a low ratio, lowered the cell membrane integrity, and reduced the M7-AMkL cell population after 24h of co-culture, while the cytotoxic effect was not significant in groups with higher E:T ratio. The cells were maintained in sterile conditions during the experiment.

Conclusions: Our findings suggest that the anti-CD41 CAR T cells are efficient for a limited time spawn and the cytotoxic effect is visible in all experimental groups with low E:T ratio.

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In vitro biological performance of 3D-printed PLA-based composites as bone scaffolds

Andreea Mariana Negrescu [a,*], **Valentina Mitran** [a], **Florin Miculescu** [b], **Aura Catalina Mocanu** [b],
Andreea Elena Constantinescu [b], **Anisoara Cimpean** [a]

a) Department of Biochemistry and Molecular Biology, Faculty of Biology, University of Bucharest, 91-95 Spl. Independentei, 050095, Bucharest, Romania;
b) Department of Metallic Science, Physical Metallurgy, University Politehnica of Bucharest, 313 Spl. Independentei, 060042, Bucharest, Romania.
**email: andreea.ne94@gmail.com*

Introduction: The positive outcome of an implant depends mainly on the functionality of the biomaterial, therefore the development of new scaffold materials with favourable mechanical and biological properties is imperative for an efficient regeneration process [1]. Due to their favourable biocompatibility and biodegradability, polylactic acid (PLA) based materials are frequently used in various medical applications, such as sutures or orthopaedic fixation devices. However, PLA alone has an insufficient mechanical strength and osteoconductivity, therefore the incorporation of different particles such as hydroxyapatite (HA) into the PLA matrix constitutes a promising strategy towards an enriched composite material [2]. Moreover, having just expanded into the biomedical field, graphene (GnP) and its derivatives continues to attract interest due to their unique characteristics and numerous efforts have been made to employ their exceptional properties for the regeneration of different tissues, particularly bone [3]. In this context, we developed 3D printed composite products, based on PLA, GnP, and naturally derived HA.

Materials and methods: The composite substrates were fabricated through the incorporation of different ratios of HA (0–50%) and GnP (0–5%) into the PLA matrix. For each sample, the mixing process required the following steps: (1) mechanical homogenization; (2) thermal homogenization at a constant temperature (~200 °C); (3) flattening of the viscous slurry between two ceramic plates to a thin lamella/film of 1 ± 0.05 mm thickness; (4) cutting the lamella into small pieces to facilitate the extrusion procedure. In the end, the extruded filaments were transformed into 3D printed plates via the Fused deposition modelling (FDM) technique. *In vitro* performance of the novel composite scaffolds was explored through direct contact assays using the MC3T3-E1 pre-osteoblasts and RAW 264.7 macrophages. The cellular behaviour of the pre-osteoblasts was assessed in terms of cell viability/proliferation, morphology and osteogenic differentiation, while the inflammatory response of the RAW 264.7 cells was investigated via cell viability/proliferation and morphological features analysis, and also through the quantification of specific inflammatory mediators and foreign body giant cells (FBFCs) formation.

Results: The results suggested that all samples, in various degrees, supported cell survival/proliferation, for both cell lines. Similar, the cytoskeletal examination revealed typical cellular morphologies; however, with significant differences in cell density (a reduced number of cells on 5% GnP and 4% GnP samples). Likewise, the differentiation study demonstrated their ability to promote new bone formation. Nonetheless, the supports led to the formation of FBGCs even in the lipopolysaccharide (LPS) absence in the next order: 5%GnP < 4%GnP < 1%GnP < 2%GnP < 3 %GnP < 1%GnP. This surprising result could be explained by the low number of cells found on the 5%GnP and 4%GnP samples as compared to the rest.

Conclusions: Taken together, our results demonstrate that all analyzed samples, with the exception of 4% GnP and 5% GnP and to a different extent, meet the strict requirements of a biomimetic material for bone tissue engineering applications.

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Novel biohybrid enriched with curcumin and quercetin displays favorable properties as potential active wound dressing for biomedical applications

Andreea-Daniela Lazăr (Popa) [a], Lea Sleiman [a,*], Maria Minodora Marin [c,d], Mădălina Georgiana Albu-Kaya [c], Marieta Costache [a,b], Sorina Dinescu [a,b]

a) Department of Biochemistry and Molecular Biology, University of Bucharest, 91-95 Spl. Independentei, 050095, Bucharest, Romania;
b) Research Institute of the University of Bucharest (ICUB), 90 Sos. Panduri, 050663, Bucharest, Romania;
c) Collagen Department, Leather and Footwear Research Institute, 93 Ion Minulescu Street, 031215, Bucharest, Romania;
d) Politehnica University of Bucharest, Advanced Polymer Materials Group, 1-7 Polizu Street, 011061, Bucharest, Romania
**email: sleiman.lea@s.bio.unibuc.ro*

Introduction: Chronic wounds require repetitive treatments, incur considerable medical costs and can lead to other dangerous medical conditions, such as diabetic ulcers, which precede amputations. Thus, much effort has been focused on developing wound dressings with intrinsic beneficial properties that could facilitate the impaired healing process of acute lesions. Among the natural agents considered for severe wound healing are phytochemicals, such as curcumin (C) and quercetin (Q). In this study, we evaluated if the addition of novel encapsulated CQ improved the properties of collagen-sericin (Coll-SS) materials in terms of biocompatibility, cell adhesion, anti-inflammatory and antioxidant effects.

Materials and methods: Coll-SS tridimensional materials, enriched or not with CQ microcapsules (Coll-SS-CQ) were developed and characterized using scanning electron microscopy (SEM). The compositions were seeded with keratinocytes from an adult human skin cell line and cultivated for up to 7 days. During biocompatibility assessment, MTT assay was used for cell viability and proliferation evaluation, LDH assay to investigate the biohybrids' cytotoxicity, and Live/Dead staining was performed to visualize the extent of live and dead cells, comparing Coll-SS-CQ to a pure Coll-SS matrix. Immunolabeling and confocal microscopy allowed observation of cytoskeleton distribution. The levels of proinflammatory mediators in response to CQ anti-inflammatory effect were evaluated after induced macrophages were put in contact with the materials, at gene and protein level. Activated macrophages exposed to no other treatment were considered positive reference in analysis. Moreover, ROS production was quantified to assess the antioxidant effect.

Results: All samples exhibited a uniform porous structure with interconnected pores, beneficial for cell growth and migration, as shown by representative SEM images. After one week of *in vitro* culture, the biohybrids presented good biocompatibility, without significant cytotoxic effects. Highest cell viability and proliferation were registered for Coll-SS-CQ material, as compared to the controls. Cytoskeleton staining revealed proper adhesion of skin cells to the substrates and highlighted an elongated cell morphology of actin microfilaments. Results indicated a clear decrease in proinflammatory cytokine levels secreted by cells in contact with Coll-SS-CQ, as well as ROS production, suggesting that the encapsulation and gradual release of CQ results in a more efficient anti-inflammatory and antioxidant response.

Conclusions: In conclusion, the novel Coll-SS biohybrid enriched with encapsulated CQ can be considered as a potential active wound dressing for biomedical applications.

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La(Nal)3 loaded PDMAEMA NPs induce HT-29 adenocarcinoma cells apoptosis in vitro

Bianca Galateanu [a,*], Alexandra Cowell [b], Valentina Uivarosi [b], Mirto Charitaki [c], Kelly Velonia [c], Ariana Hudita [a]

a) University of Bucharest, 90 Panduri Avenue, Bucharest, Romania;
b) University of Medicine and Pharmacy Carol Davila from Bucharest, 37 Dionisie Lupu Street, Bucharest, Romania;
c) University of Crete, Crete, Greece;
**email: bianca.galateanu@bio.unibuc.ro*

Introduction: Recent statistics rank colorectal cancer (CRC) as the second cause of cancer-related deaths and third as incidence [1] and highlight the emerging need to improve the currently available therapeutic approaches. In the context of increasing resistance and dose-limiting side effects to traditional chemotherapy, a current strategy in bringing the activity of 'older' drugs back into clinical use for purposes that are outside the scope of the original medical indication (drug repositioning) [2]. With this respect, we intend to shift the 'old' antibacterial quinolones to anticancer compounds via coordination with appropriate metal ions such as La. In this study, we investigated the anticancer activity on HT-29 adenocarcinoma cells of the complex La(Nal)3 (HNal = nalidixic acid) and of the complex-loaded poly[2-(N,N-dimethyl amino) ethyl methacrylate (PDMAEMA) based nanoparticles(NPs).

Materials and methods: The IC_{50} dose of the $La(Nal)_3$ – loaded PDMAEMA NPs on HT-29 adenocarcinoma cells was previously determined by using the MTT spectrophotometric assay. This dose was further used to investigate the pro-apoptotic potential of the pristine and $La(Nal)_3$ – loaded PDMAEMA NPs. Briefly, HT-29 adenocarcinoma cells were seeded at an initial cell density of 5×10^3 cells/cm^2 in 6 well plates and allowed 24h to adhere to the culture surface in standard conditions of culture (37 ^{0}C, humidified atmosphere and 5% CO_2). Then, the monolayers were treated with pristine NPs, $La(Nal)_3$ – loaded PDMAEMA NPs and $La(Nal)_3$ and incubated for another 6h and 24h. An untreated control monolayer was kept for each time point. Apoptosis was investigated by anexin-V/PI test using flow cytometry (Cytoflex, Beckman Coulter)

Results: Our results show that both the control and the cells treated with pristine NPs remained viable and negative for anexin V and PI, while the cells treated with $La(Nal)_3$ and $La(Nal)_3$ – loaded PDMAEMA NPs entered apoptosis after 6h of treatment.

Conclusions: In conclusion, the novel $La(Nal)_3$ – loaded PDMAEMA NPs exert pro-apoptotic effects *in vitro* and could be further used for *in vivo* studies to investigate their bio distribution and delivery efficacy.

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Novel photosensitizing methylene blue analogues for antimicrobial photodynamic therapy

Bianca Stoean [a,*], **Iulia Lupan** [b], **Luiza Găină** [a]

a) Research Center on Fundamental and Applied Heterochemistry, Faculty of Chemistry and Chemical Engineering, Babeş-Bolyai University, 11 Arany Janos str., RO-400028 Cluj-Napoca, Romania;
b) Department of Molecular Biology and Biotechnology, Babes-Bolyai University, Cluj-Napoca, Romania;
**email: bianca.stoean@ubbcluj.ro*

Introduction: Photodynamic therapy (PDT) is based on the principle of administering a photosensitizer, followed by irradiation with visible light (using the wavelength specific to the absorption of the photosensitizer molecule). In the presence of molecular oxygen in the cells, the photosensitizer forms cytotoxic species ($^1O_2$-oxygen singlet, free radicals-ROS) that result in cell death through various mechanisms, such as apoptosis, angiogenesis or autophagy. Used in the last two decades as a treatment against malignant and benign tumors, photodynamic therapy seems to be a good alternative in the fight against bacteria that have become resistant to existing antibiotics [1].

Materials and methods: Broth dilution method were performed in order to test the inhibition effects of tested MB compounds against *Escherichia coli ATCC 25922; Staphylococcus aureus ATCC 25923; Pseudomonas aeruginosa ATCC 27853; Deinococcus radiodurans* (DSM no. 20539). The tested bacterial strain was cultivated in Mueller-Hinton Broth (*P. aeruginosa* and *S. aureus*) and Luria Broth (*E. coli*) at 37°C or TGY medium (*D. radiodurans*) at 28°C. Minimum inhibitory concentration (MIC) was determined for both the scenario where methylene blue (MB) analogues were not irradiated and the case where the compounds were irradiated with 660 nm wavelength light of 1,2 mW power coming from a LED lamp (M660L4-C1) leading to the singlet oxygen formation.

Results: For all studied methylene blue analogues, the minimum inhibitory concentration for all tested bacterial strains (gram-positive and gram-negative) were remarkably lower with 660 nm light LED irradiation, demonstrating the efficacy of using photodynamic therapy against resistant bacteria.

Conclusions: In this work we examine the ROS production capacity and the potential for use in antimicrobial photodynamic therapy for a series of MB analogues recently reported by our group [2] covering auxochromic alteration by steric and electronic factors affecting the position of their absorption wavelength maxima, aggregation and hydrophilic/lipophilic balance.

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Building a 3D printed microscope – the OpenFlexure@BioIASI project

Ciurlic Maria Paula [a], Colpoș Andreea Monica [a], Ștefan Marius [a], Mihășan Marius [a]

a) BioActive research group, Department of Biology, Alexandru Ioan Cuza University of Iași, Romania
**email: ciurlicmp@gmail.com*

Introduction: The OpenFlexure project (https://openflexure.org) [1, 2] aims to provide an open-source, 3D-printed microscope with applications in both medical and teaching environment. OpenFlexure@BioIASI is a student-based project that kicked off in 2021 with the goal of building and evaluating the real costs of a 3D printed OpenFlexure microscope and its applications in the practicals taking place at the Department of Biology, Alexandru Ioan Cuza University of Iași.

Materials and methods: All parts required were sourced from local and overseas distributors (Aliexpress and alike) at a total cost of about 200 euros. All objectives were sourced from old or not working microscopes. All printed parts were fabricated from PLA using an Creality Ender-5 printer at a total cost of 5 euro.

Results: Following the instructions available, a fully motorized, single objective microscope was built which uses a Raspberry Pi camera to acquire images and a Raspberry Pi single board computer running the specialized Raspbian OpenFlexure Lite OS to control the motors. The microscope was configured to generate its own Wi-Fi network and hence a simple laptop with a browser is all that is required to control it. Its optical performance was so far tested in different settings and it was shown that it provides images that are comparable with other microscopes such as: Leica DM 1000 LED, Lacerta BIM 151 T-LED, Optika B-157R-PL, Optika B-350 PL and Olympus BX41. Some drawbacks were nevertheless recorded, such as a low power light supply, poor condenser lenses and low overall robustness of the build.

Conclusions: In a controlled environment, the 3D printed microscope could replace professional microscopes and provide good-enough images.

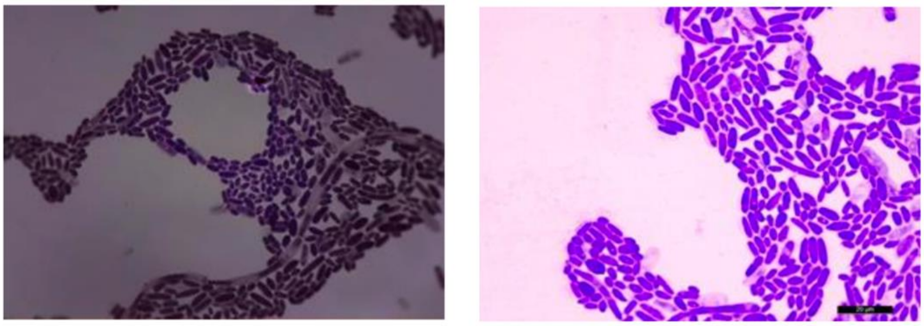

Figure 1. *Candida albicans* cells view under the Openflexure microscope (left) and a Leica DM 1000 microscope (100x)

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MDS cells maturation induced by ATRA differentiating agent in myelodysplastic syndromes

Diana Cenariu [a], Adrian Bogdan Tigu [a], David Kegyes [a], Mareike Peters [a], Jon Thor Bergthorsson [f], Victor Greiff [g], Cenariu Mihai [e], Gabriel Ghiaur [a,h] , Ciprian Tomuleasa [a,b,c,d]

a) Medfuture Research Center for Advanced Medicine, Iuliu Hatieganu University of Medicine and Pharmacy Cluj Napoca, Cluj Napoca, Romania
b) Department of Hematology, Iuliu Hatieganu University of Medicine and Pharmacy Cluj Napoca, Cluj Napoca, b) omania
c) Department of Hematology, Ion Chiricuta Clinical Cancer Center Cluj Napoca, Cluj Napoca, Romania
d) Department of Chemotherapy, Ion Chiricuta Clinical Cancer Center, Cluj Napoca, Romania
e) Univ Agr Sci & Vet Med, Dept Anim Reprod & Reprod Pathol, Cluj Napoca 400372, Romania
f) Univ Iceland, Biomed Ctr, Sch Hlth Sci, Stem Cell Res Unit, Reykjavik, Iceland
g) Univ Oslo, Dept Immunol, Oslo, Norway
h) Johns Hopkins University, 1650 Orleans St. Room 243, Baltimore, MD 21231, USA
**email: diacenariu@gmail.com*

Introduction: Myelodysplastic syndromes (MDS) are heterogeneous hematopoietic stem cell (HSC) disorders characterized by bone marrow (BM) dysplasia and peripheral cytopenias [1, 3]. CyP26 activity in the BME protects normal and malignant HSCs from ATRA-induced differentiation and contributes to drug resistance. [1, 2]. Malignant cells can hijack control of stromal CYP26 activity to create permissive microenvironments [3].

Materials and methods: In vitro cultivation of MDS92 cells and OP9 cells, treated with different ATRA concentrations (10e-9M, 3x10e-9M, 10e-8M, 3x10e-8M, 10e-7M) to induce differentiation. Talarozol (R115866), an inhibitor that blocks CyP26 was added to the ATRA treatment in the experimental design, at 1nM cc-tion. Flow cytometry evaluation. The processed experimental samples are labelled with 4 antibodies: CD45PerCP-Cy5.5: CD38APC-H7: CD11b APC: GlyA-PE and the mean flourescence intensity is calculated. Colony forming units are performed to evaluate and validate the maturation of MDS cells following ATRA treatment, by counting the number of colonies formed in each plate. 2.000 cells from each combination MDS92 no stroma and MDS92 co-cultivated with stromal OP9 on 6 well plates on methylcellulose for 14 days, have been counted.

Results: CD45 staining isolates the leucocyte population out of the analyzed cells; from here, specific antibodies that prove maturation of MDS92 cells are used: CD38, CD11b and Gly A. There is a shift in the positive population of mature cells dependent of the ATRA treatment concentration. Best results are observed at 10e-8M ATRA. The number of colonies formed is counted in triplicate for all wells and compared in Graph Prism 8.0. The number of colonies formed decreases, once the differentiation of MDS92 cells is induced due to the retinoic acid treatment, in the presence/absence of stroma.

Conclusions: Since myelodysplastic cells mature slower in the presence of stroma than those grown without stroma by the addition of Talarozol to the experimental design we could prove that R115866 blocks CyP26, thus therapy resistance can be eliminated in vitro. The ATRA inactivation is stronger on MDS92 in the presence of stroma OP9, which proves that Cyp26 is involved in limiting the production of the active ATRA.

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3D tumor spheroids as a model to unriddle the complexity of tumor microenvironment chemoresistance

Giorgiana Negrea [a*], Szilvia Meszaros [b], Stefan Drăgan [b], Vlad-Alexandru Toma [b,c], Bogdan Dume [a], Valentin-Florian Rauca [b], Laura Pătraș [b], Emilia Licărete [b], Manuela Banciu [b], Alina Sesărman [b]

a) Doctoral School in Integrative Biology, Faculty of Biology and Geology, "Babes-Bolyai" University, Cluj-Napoca, Romania;
b) Department of Molecular Biology and Biotechnology, Center of Systems Biology, Biodiversity and Bioresources, Faculty of Biology and Geology, "Babes-Bolyai" University, Cluj-Napoca, Romania;
c) Department of Experimental Biology and Biochemistry, Institute of Biological Research, Branch of NIRDBS Bucharest, Cluj-Napoca, Romania
**email: giorgiana.negrea@ubbcluj.ro*

Introduction: Recent oncology advances emphasize the tumor microenvironment (TME) critical role in drug resistance. Therefore, accurate models reflecting TME complexity are essential. 3D models offer greater tumor heterogeneity compared to 2D cell cultures, making them a pioneering tool for cancer research. Our aim was to create 3D in vitro models with B16.F10 murine melanoma cells combined with endothelial cells (2H11) and/or primary macrophages, to mimic the chemoresistant melanoma microenvironment, and to validate them using doxorubicin.

Materials and methods: Macrophages were isolated from C57BL6 mice bone marrows. Spheroids were created using the liquid overlay technique, co-culturing B16.F10 murine melanoma cells with endothelial cells or macrophages in non-adherent 96-well plates with 1.5% commercial extracellular matrix (ECM). Characterization of the 3D cocultures included morphology analysis by light microscopy, as well as immunohistochemistry using specific antibodies for component cells. To assess the models' ability to mimic melanoma microenvironment chemoresistance, spheroids were treated with DOX (IC30), a chemotherapeutic drug to which melanoma patients fail to respond. Parameters like HIF-1α, VEGF,and leptin expression, total antioxidant capacity, and peroxidase activity were evaluated using western blot, protein micro-array, and spectrophotometry.

Results: The developed models displayed some of the chemoresistance traits. After DOX treatment, the 3D coculture with B16.F10 melanoma cells and macrophages showed significant increases in total antioxidant capacity (TAC) and catalase activity ($p<0.05$) compared to the control group (untreated spheroids). Immunohistochemistry analysis indicated no changes in peroxidase activity ($p>0.05$) in DOX-treated spheroids compared to control. Moreover, the chemoresistant profile persisted when B16.F10 melanoma cells were 3D cocultured with endothelial cells and treated with DOX, as key angiogenesis promoters (HIF-1α, VEGF, and leptin) remained unchanged compared to the control group ($p>0.05$). These findings demonstrate that the cells involved in the 3D coculture models, exhibit a deliberate mechanism to evade substantial molecular alterations when exposed to DOX treatment, suggesting a strategic approach in preserving protumor processes crucial for driving tumor growth.

Conclusions: Our results indicate the reliability of our models in reflecting chemoresistance characteristics of the melanoma microenvironment. To better understand the complex cellular interactions driving melanoma's chemoresistant profile, further investigation with additional cell types and analysis of other key parameters is needed.

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Peptide-based therapeutics

Lucian Copolovici [a*], Andreea Gostăviceanu [a,b], Simona Gavrilaş [a], Dana G. Radu[a], Mihaela Dochia [a], Cristian Moisa [a], Andreea Ioana Lupitu [a], Dana Maria Copolovici [a]

a) "Aurel Vlaicu" University of Arad, Faculty of Food Engineering, Tourism and Environmental Protection and Institute of Technical and Natural Sciences Research-Development-Innovation of "Aurel Vlaicu" University, 310010, Elena Dragoi Street, nr. 2, Arad, Romania
b) University of Oradea, Biomedical Sciences Doctoral School, Universității Street, Nr.1, 410087, Oradea, Romania
**email: lucian.copolovici@uav.ro*

Introduction: Traditional treatments for cancer have very bad side effects. Peptide-based therapy as a complementary choice for treating cancer has shown promising results because it is selective, and is safe [1,2]. The goal of this study was to explain the different ways that peptides are used in cancer therapy, as well as the engineering techniques that are used to improve the effectiveness of peptides, and to look at the current state and future trends of publications about anticancer peptides.

Materials and methods: For the bibliometric study, we searched articles published in PubMed, ScienceDirect, and Web of Science, Clarivate, in the last two decades [1,2,3,].

Results: Information was gathered about how peptides work in anticancer treatment. Anticancer peptides may get rid of cancerous cells in more than one way. Engineering techniques have been used successfully to improve the effectiveness of peptides by making them more bioavailable. The bibliometric study depict how important anticancer peptides have become in the treatment of cancer in the last 20 years.

Conclusions: Biopharmaceutical peptides have led to new ways to treat and prevent many diseases, including cancer. Peptide-based therapeutics have opened up new ways to control drug delivery and improve clinical results in cancer by using innovative peptide-based assembly. Like some common polymeric formulations, these macromolecules may be able to greatly slow the rate at which a drug is released. Peptides, unlike many polymeric formulations, can be made to fit a specific sequence by using genetic engineering. This makes them an interesting option to traditional formulations. Combinations of peptide-based strategies and conventional therapies are being investigated for their potential synergistic effects in the development of anticancer drugs.

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Cell behaviour modulation by new polydimethylsiloxane (PDMS) modified interfaces

Madalina Icriverzi [a#]*, **Paula Ecaterina Florian** [#a], **Anca Bonciu** [b], **Nicoleta Dumitrescu** [b],
Laurentiu Rusen [b], **Valentina Dinca** [b] **Anca Roseanu** [a]

a) Institute for Biochemistry of Romanian Academy, 060031 Bucharest, Romania;
b) National Institute for Laser, Plasma and Radiation Physics, 077125 Magurele, Romania
**email: *mradu@biochim.ro; #equal contribution*

Introduction: Different strategies for improved efficiency of implanted devices are developed. Surface texturing and chemical modifications are important strategies to allow medical-devices to improve biocompatibility and also minimizing adverse responses. Here we present our results regarding polydimethylsiloxane (PDMS) modified interfaces, their physical-chemical characteristics and *in vitro* effect on cell behaviour [1, 2].

Materials and methods: Matrix-Assisted Laser Evaporation (MAPLE) method [3] was used to create the newly proposed functionalized scaffold and physical-chemical characteristics were evaluated by Scanning Electron Microscopy, Atomic Force Microscopy, Contact angle, Surface energy, Fourier Transform Infrared Spectroscopy and X-ray Photoelectron Spectroscopy. Cell adhesion, proliferation and morphology of cells grown on Polydimethylsiloxane (PDMS) surfaces were evaluated in in vitro studies, using human macrophages and fibroblasts, cells paying an important role in foreign body reaction.

Results: Physical and chemical properties of the new coatings revealed that the MAPLE technique has the advantage of achieving homogeneous, stable and moderate hydrophilic thin layers onto hydrophobic PDMS. In addition, the proposed approach does not require any pre-treatment, therefore avoiding the major disadvantage of hydrophobicity recovery. Statistically significant reduction of the adhesion and proliferation of human macrophages and of human fibroblasts grown on PDMS with modified surfaces compared to unmodified scaffold was revealed by *in vitro* biological investigations. These findings evidence the capacity of the newly PDMS modified surfaces to modulate cell behaviour and thus to overcome undesired cell responses such as inflammation and fibrosis.

Conclusions: Our data highlighted the potential for the new PDMS modified interfaces obtained by MAPLE to develop PDMS-based implants able to mitigate foreign body response and thus to be used in the biomedical field.

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Biodegradable chitosan-based films for biomedical applications

Mariana-Adina MATICA [a*], **Alexandra NĂSTASIE** [b], **Alexandra TUDOR** [b], **Timea KELEMEN** [b], **Ramona ALICU** [b], **Diana-Larisa ROMAN** [b, c]

a) Department of Chemistry-Biology, Institute for Advanced Environmental Research, West University of Timisoara, 4C Oituz, 300086, Timisoara, Timiș, Romania
b) Department of Biology-Chemistry, Faculty of Chemistry, Biology, Geography, West University of Timisoara, 16 Pestalozzi, 300115 Timisoara, Timiș, Romania
c) Advanced Environmental Research Laboratories, West University of Timisoara, 4A Oituz, 300086, Timisoara, Timiș, Romania
**email: mariana.matica@e-uvt.ro*

Introduction: Chitosan is a cationic polymer, widely found in nature, and used in biomedical application due to its numerous advantages such as biodegradability, biocompatibility, antimicrobial and wound healing properties, as well as low toxicity. The film-forming capacity of chitosan makes it a suitable polymer for wound dressing development. The use of chitosan combined with antimicrobial peptides (AMPs) is a common practice adopted in order to overcome the increase of antibiotic resistance to conventional antibiotics. Nisin is a polycyclic cationic peptide classified as a Type A (I) lantibiotic molecule and a GRAS (Generally Recognized As Safe) molecule, mainly used in food industry, but recent approaches indicate its use biomedical fields, as an alternative to common antibiotics. The purpose of this study was to design chitosan-based films with enhanced antibacterial effect against most common pathogenic bacteria, using nisin as a potential antimicrobial compound.

Materials and methods: A medium molecular weight chitosan was used to prepare chitosan-based films containing different concentration of nisin (5 mg/mL and 10 mg/mL). Chitosan films were prepared via solvent casting method and glycerol was added as a plasticizer. Physico-chemical properties of the films were determined, as well as the microbiological properties, using most common pathogens strains (*Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*). Moreover, biodegradable studies were carried out by testing chitosan-based films degradation in soil and in order to observe the impact of the polimeric materials, the total microbial count and enzymatic activity of soil microbiome was analyzed.

Results: Chitosan-based films are suitable materials for the sustained delivery of antimicrobial compounds. The results have shown that the release of nisin from chitosan matrix happens at a slow and sustained rate, at a pH value similar to the most common wound environment. Such properties such as swelling, water-vapor permeability and optical properties of the films indicate that the biomaterials are suitable as wound dressing starting material. Moreover, the microbial permeation studies and microbiological analysis reveal that the addition of nisin increases the antibacterial effect of the films, while the biodegradability studies show that these types of materials are safe for the environment.

Conclusions: Chitosan plays an important role in biomedical application as it offers a safer and environmentally friendly alternative în creating drug-delivery platforms, with slow and sustained drug release. The use of AMPs in order to enhance the pristine antibacterial effect of chitosan, can be favorable and can help overcome the main drawback represented by the use of conventional antibiotics. Moreover, the availability of chitosan as a waste byproduct generated by the seafood processing industry is a topic that lines up with the interest of current scientific research, which is to enhance exploitable resources in the context of the circular and sustainable economy.

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Development of a 3D Model for Immunotherapy Evaluation in Melanoma

Marta-Szilvia Meszaros [a]; **Giorgiana-Gabriela Negrea** [b]; **Vlad-Alexandru Toma** [a,c]; **Valentin-Florian Rauca** [a]; **Emilia Licarete** [a]; **Laura Patras** [a], **Manuela Banciu** [a]; **Alina Sesarman** [a]

a) Department of Molecular Biology and Biotechnology, Center of Systems Biology, Biodiversity and Bioresources, Faculty of Biology and Geology, “Babes-Bolyai” University, Cluj-Napoca, Romania
b) Doctoral School in Integrative Biology, Faculty of Biology and Geology, “Babes-Bolyai” University, Cluj-Napoca, Romania
c) Department of Experimental Biology and Biochemistry, Institute of Biological Research, Branch of NIRDBS Bucharest, Cluj-Napoca, Romania
**email:* alina.sesarman@ubbcluj.ro

Introduction: Previous research has shown that immunotherapies are a promising treatment opportunity for many types of cancer. Melanoma is regarded as a great candidate for immune checkpoint inhibitors due to the immunogenicity caused by a high tumor mutational burden. However, some melanoma patients do not respond well to immunotherapy. To address this, improved preclinical models are needed, particularly 3D models that accurately represent the tumor microenvironment's elements, spatial arrangement, and treatment response. This study aimed to generate heterocellular spheroids consisting of B16.F10 melanoma cells, dendritic cells, and $CD8^+$ T cells to investigate the combined effects of anti-PD-L1 antibody and curcumin, as an immunotherapy strategy.

Materials and methods: Spheroids were formed using the "liquid overlay" technique by seeding B16.F10 melanoma cells, bone marrow-derived dendritic cells, and $CD8^+$ T cells isolated from C57BL6 mice's lymph nodes, onto non-adherent plates[1]. The spheroids' morphology was examined using light and fluorescent microscopy. Immunohistochemistry was used to assess the presence of immune cells within the spheroids using specific antibodies. The acid phosphatase assay determined the impact of the immunomodulatory treatments on the viability of the spheroid cells[2]. Additionally, the western blot technique was employed to investigate the treatment's influence on HIF-1α, pNF-kβ p65, pAP-1 c-Jun, and Akt expression levels[3].

Results: The administration of curcumin resulted in decreased cell viability within heterocellular spheroids. The IC_{50} valueof curcumin was determined to be 15 µM. This effect was accompanied by a decrease in the expression levels of HIF-1α, while no significant changes were observed in pNF-kBp65 or pAP-1c-Jun protein expression levels. Notably, when combined with anti-PD-L1 antibodies, a concentration of 25 µM curcumin led to nearly complete inhibition (almost 100%) of cell viability in heterocellular spheroids.

Conclusions: In conclusion, our team has developed a new 3D cell culture platform which enables the incorporation of diverse cell types and facilitates testing with a broad spectrum of immunotherapeutics.

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Miniaturized Electrochemical Biosensor for Troponin I Protein - Cardiac Biomarker Detection

Mihnea Moruz [a,*], **Andreea Campu** [b], **Simion Astilean** [b], **Monica Focsan** [b]

a) Faculty of Physics, 'Babeș-Bolyai' University, 1 M. Kogălniceanu Str., 400084, Cluj-Napoca, România;
b) Nanobiophotonics and Laser Microspectroscopy Center, Interdisciplinary Research Institute in Bio-Nano-Sciences, 'Babeș-Bolyai' University, 42 T. Laurian Str., 400271, Cluj-Napoca, România;
**email: moruz.mihnea@yahoo.com*

Introduction: The cardiovascular category of diseases, a major contributor to global mortality, accounts for 31% of worldwide deaths, with an expected 10% increase in affected individuals over the next decade.[1] Researchers have focused on studying biomarkers, such as Troponin I, to prevent acute myocardial infarction, which is recognized by the European Society of Cardiology as the most suited.[2]

Materials and methods: In the current work, the development of a novel and efficient electrochemical immunosensor device was targeted, through the synergistic combination of a sensor that integrates plasmonic electrodes of desired shape and size on paper with a portable electrochemical system. The electrodes realised by deposition of thin gold films on a paper substrate were characterized both optical and morphological. Then, the electrodes were placed on a PDMS substrate, confering both stability, and elasticity to the fully realised device. The validation of the device to detect Troponin I was confirmed using Surface-Enhanced Raman Spectroscopy (SERS) and Cyclic Voltammetry (CV) methods.

Results: A 90 nm thick continuous gold layer, characterized by a rugosity of 3.2 nm, was used as active area for detection purposes. In order to calibrate the detection method using Differential Pulse Voltammetry (DPV), we tested different concentrations of Troponin I in a biologically simulated environment and finally in plasma samples from patients.

Conclusions: In conclusion, it is anticipated that a miniaturized and electrochemical nanodevice will be provided, which is adapted for specific portable detection of the cardiac biomarker Troponin I. Furthermore, the device can be easily customized and optimized for a wide range of medical and environmental applications of interest. The unique properties of the proposed immunosensor can potentially offer a real advantage in the early diagnosis of cardiovascular diseases, thereby positioning our device as an excellent candidate for potential technology transfer.

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Develop a genetic engineering tool for *Paenarthrobacter nicotinovotans* ATCC 49919

Munteanu Iustin-Tiberius[a*] **and Mihășan Marius**[a]

a) BioActive Reseacrh Group, Faculty of Biology,"Alexandru Ioan-Cuza" University of Iasi, Blvd Carol I no. 20A, Romania
**email:* tibimunteanu897@gmail.com

Introduction: *Paenarthrobacter nicotinovorans ATCC 49919* is a nicotine degrading microorganism (NDM) with biotechnological potential for the production of compounds of industrial and pharmaceutical importance like 6-hydroxy-L-nicotine, methylamine, succinic acid or gamma-amino-butyric-acid. The nicotine catabolism is related to the presence of pAO1 plasmid (1). In the present study we aim to develop a genetic engineering tool based on the CRISPR system that would allow fast and easy editing of the *P. nicotinovorans* genome to increase its biotechnological potential. Our aim is to inactivate or drastically reduce the expression of 6-hydroxy-L-nicotine oxidase (*6hlnO*), the key enzyme that catabolizes the conversion of 6-hydroxy-L-nicotine (6-HLN) to 6-hydroxy-methylmyosmine (6-HMM).

Materials and methods: Plasmid DNA have been isolated using QIAprep Spin Miniprep Kit. CRISPR-Cpf1 genes from pJYS3-ΔcrtYF plasmid have been isolated by PCR using the fallowing primers set: For: TCCGACGTCGTCGACTTTGCTGTTTACAATTAATCATCGTGTGG; Rev:ACCACTAGTCCTAGGTTTTTGACAGCTAGCTCAGTCCT and cloned into the DraI linearized pART2 vector using Gibson Assembly (NEBuilder HiFi DNA Assembly Master Mix). Positive recombinant plasmids were selected fallowing digestions with SpeI and ApaLI.

A 20 bp spacer targeting the gene of interest was obtained by annealing two 5′ phosphorylated synthetic oligonucleotides (For: GAAAAAGTTGCAGCATCCAAAGCG and Rev: AAACCGCTTTGGATGCTGCAACTT) by incubating at 95°C for 3 minutes and then gradually cooling the mixture by 0.010°C every 10 seconds for 2 hours. The spacer was cloned using Golden-Gate assembly (NEBridge Golden Gate Assembly Kit BsaI-HF$^{®}$v2) into pCasiART. Positive colonies have been selected by blue-white screening (2). Positive recombinant plasmids have been electroporated into the *P. nicotinovorans* fallowing the protocol described by Zhang et al. (3). DNA concentrations were routinely evaluated using agarose gel electrophoresis and a Quantus^{TM} Fluorometer.

Results: Two distinct approaches have been used to inactivate the gene of interest. One is based on the CRISPR-Cpf1 system and aims to completely knock-out the *6hlnO* gene by deleting it. Genes containing the CRISPR-Cpf1 system from plasmid pJYS3-ΔcrtYF (4) (AddGene #85542) have been transferred into the pART2 plasmid using Gibson assembly, resulting the pART2-Cpf1 plasmid. We are in the process of targeting the CRISPR-Cpf1 system for *6hlnO* by cloning a crRNA sequence and corresponding protospacers into the pART2-Cpf1.

The second approach is based on the CRISPR/dCasi9 system and allows partial and controlled inactivation of the *6hlnO* gene transcription. Thus, the CRISPR/dCasi9 system has been targeted to the gene of interest by cloning a 20 bp spacer with the sequence complementary to the *6hlnO* gene into the pCasiART plasmid using the Golden-Gate assembly method and the pCasiART-Δ6hlnO have been obtained (2). The resulting plasmids have been introduced into *Paenarthrobacter nicotinovorans* by electroporation.

Experiments are underway for evaluating the efficiency of *6hlnO* gene inactivation of the two constructs: pART2-Cpf1-Δ6hlnO and pCasiART-Δ6hlnO. For this, cultures in liquid-broth will be performed and samples at regular intervals will be analyzed by HPLC for quantification of nicotine and 6HLN levels.

Conclusions: At this stage, pCasiART-Δ6hlnO and pART2-Cpf1 plasmids were obtained, and tests are underway for evaluating the efficiency of these genetic engineering tools.

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Carotenoid loaded nanocarriers - preparation and evaluation

Patricia Muntean [a,*], **Adela Pintea** [a], **Andrea Bunea** [a], **Francis V. Dulf** [a], **Monica Focşan** [b], **Dumitriţa Rugină**[a]

a) University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Calea Mănăştur 3-5, 400372, Cluj-Napoca, Romania;
b) Nanobiophotonics and Laser Microspectroscopy Center, Interdisciplinary Research Institute in Bio-Nano-Sciences, Babes-Bolyai University, 42 Treboniu Laurean, 400271 Cluj-Napoca, Romania;
**email: patricia-andreea-lia.muntean@usamvcluj.ro*

Introduction: Zeaxanthin is a xanthophyll carotenoid that is widely distributed in tissues and is the primary carotenoid in the eye lens and retinal macular region. Zeaxanthin has antioxidant effects and can be employed as a bioactive molecule because of its favorable effects on human health. [1] Plants, bacteria, and microalgae all create this beneficial pigment, but because of their hydrophobicity, their bioavailability and effectiveness in biological systems is limited [2]. Moreover, zeaxanthin has a low thermal and photostability. As a consequence, it is essential to overcome such problems to gain access to the high proficiency of beneficial capacities of zeaxanthin. Nanoencapsulation provides an advantageous environment for the bioactive contents of foods to be protected from stressful conditions [3].

Materials and methods: Zeaxanthin with a 95% purity was obtained from plant material by solvent extraction and purification using TLC and column chromatography. Four vegetal oils (coconut MCT, olive, sunflower, and linseed) were profiled for fatty acids composition using GC-MS and for carotenoids using C30-HPLC-PDA. In the presence of a 1% emulsifier (Tween 20), ultrasonic emulsification was used to produce zeaxanthin O/W nanoemulsions (NE) (1:9 v/v) loaded with carotenoids (0.05 mg/ml). DLS, z-potential, and SEM were used to characterize NEs, which were then subjected to a 30-day stability study under various conditions (temperature, salt content, and pH). Temperature stress test was performed by storing the NEs in vials at various temperatures (4, 20, 37, 50, and 60°C) in the dark. NEs were diluted with increasing amounts of aqueous NaCl solution to produce a series of samples with the same particle dosage but different salt concentrations (0, 25, 50, 100, and 250 mM NaCl). HCl 0.1N and NaOH 1N solutions were used to adjust the pH to the desired range (pH 3 to 8). Except for the temperature stability test, all samples were stirred before being transferred into vials and stored in a dark place at room temperature.

Results: Vegetal oils have a high rate of carotenoids incorporation into nanoemulsions (up to 98%). The surface was negatively charged (zeta potential ranging from -2 to -30 mV) and the PDI was less than 0.3. Depending on the oil type, average diameters ranged from 100 to 300nm. The stability investigation indicated that under specific stress situations, NEs might become unstable. Temperature was shown to be the most stressful factor; the NEs became unstable in the first few days after the experiment started, beginning from 37℃. High concentrations of NaCl starting at the halfway point of the experiment were the only concentrations that had an impact on NEs stability. The stability of NEs loaded with zeaxanthin was not significantly impacted by pH.

Conclusions: According to preliminary testing, the NE in which linseed oil was used as the lipidic matrix was the most stable, followed by coconut MCT, sunflower and olive oil based NEs.

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Synthesis, characterization and in vitro evaluation of a new smart responsive pNIPAM –based biopolimer with antimicrobial and antitumoral activity

Paula Ecaterina Florian [a,*#], Madalina Icriverzi [a#], Anca Bonciu [b], Luminita Nicoleta Dumitrescu [b], Diana Pelinescu [c], Cristian Munteanu [a], Livia Elena Sima [a], Valentina Dinca [b], Laurentiu Rusen [b#], Anca Roseanu [a#]

a) Institute of Biochemistry of the Romanian Academy, Splaiul Independentei 296, 060031 Bucharest, Romania;

b) National Institute for Lasers, Plasma, and Radiation Physics, 409 Atomistilor Street, 077125 Magurele, Romania;

c) Faculty of Biology University of Bucharest, Department of Genetics, Intrarea Portocalelor 1-3, Sector 6, Bucharest, Romania

**email: florian@biochim.ro; #equal contribution*

Introduction: Obtaining new smart biomaterials with pH and thermo-sensitive properties relevant for applications in implantology and tissue engineering domains represents one of the main challenges in biomedical field. The aim of our work was to investigate whether Poly-Nisopropylacrylamide-butyl acrylat (pNIPAM-BA) co-polymer porous coatings prepared by Matrix-Assisted Pulsed Laser Evaporation (MAPLE) technique and subsequently loaded with cationic peptides belonging to antimicrobial peptide family resulted in a new and more efficient scaffold with antibacterial and anticancer action.

Materials and methods: The influence of various solvents and laser fluences on the physical-chemical characteristics of the pNIPAM-BA based coatings were evaluated by atomic force microscopy, scanning electron microscopy (SEM), contact angle measurements, Fourier-transform-IR spectroscopy and X-ray photoelectron spectroscopy. The antimicrobial effect of the new developed biomaterials was investigated using clinical relevant bacteria such as *Staphylococcus aureus* (Gram+), a human pathogen typically found in post-surgical infections, *Escherichia coli* (Gram-) and *Pseudomonas aeruginosa*, a highly antibiotic resistant pathogen associated with nosocomial infections. The capacity of pNIPAM-BA scaffolds, loaded or unloaded with different active compounds, to inhibit cell proliferation and cell cycle progression as well as to induce an apoptotic effect was investigated *in vitro*, in human normal cells and melanoma cell lines.

Results: pNIPAM-BA porous polymeric platforms were obtained by MAPLE technique using DMSO as solvent and 72 kpulses fluence. Our study revealed that the pNIPAM-BA coatings featuring nanopores were suitable substrates for incorporation of cationic peptides exhibiting antibacterial and anti tumoral properties. The embedded scaffolds with peptides demonstrated the capacity to impair *in vitro* bacterial growth and to significantly reduce cancer cell proliferation, by increasing the number of apoptotic and necrotic cells.

Conclusions: Our data are promising considering how a smart stimuli-responsive scaffold loaded with active compounds could point towards a positively impact for treatment of key pathological conditions, such as cancer and bacterial infection.

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A new design of Hepatitis C Virus E2 chimeric protein oligomer as a vaccine candidate

Raed Nachawati[*], **Paula Florian, Anca Roseanu, Costin-Ioan Popescu**

Institute of Biochemistry of the Romanian Academy, 296 Splaiul Independentei,
Bucharest, Romania;
**email: nachawati.raed98@gmail.com*

Introduction: Hepatitis C virus (HCV) is a positive sense, single-stranded RNA virus that causes liver disease, including liver cirrhosis and hepatocellular carcinoma. Around 71 million people worldwide suffer from chronic HCV infection. Direct-acting antiviral agents developed in recent years can cure HCV infection in most patients, but they do not prevent reinfection and are costly in some parts of the world. Moreover, a vaccine against HCV is not yet available. The viral envelope glycoprotein E2 is the main target of neutralizing antibodies and has been used as the basis for vaccine design strategies. Most vaccine development efforts have been focusing on monomeric forms of HCV E2 but with little success. It was previously reported that multivalent display of HCV E2 antigens may improve the potency of the humoral immune response. In this study, we present a new strategy to potentially enhance the immunogenicity of E2 by designing a fusion protein comprising E2 and an oligomerization motif which self-assembles into trimers. Our hypothesis is that the oligomerization domain could also increase the uptake of the fusion protein in antigen-presenting cells by receptor-mediated endocytosis.

Materials and methods: A naturally occurring oligomerization motif was genetically fused to the HCV E2 ectodomain (residues 405-661). HEK293T cells were transfected with the construct encoding the fusion protein, followed by protein purification via affinity chromatography using Ni-NTA resin. The identity, purity and oligomerization profile of the recombinant protein were evaluated by SDS-PAGE followed by Coomassie Blue staining and Western Blot. For large-scale protein production, the fusion protein was expressed in Expi293 cells following the manufacturer's instructions. The recombinant protein was then labeled with Alexa Fluor 647, and its internalization in macrophages differentiated from the monocytic cell line THP-1 pretreated or not with chlorpromazine (a clathrin-mediated endocytosis inhibitor) was assessed by FACS analysis.

Results: A fusion protein between HCV E2 and an oligomerization domain was expressed and purified in a mammalian recombinant system. Western Blot analysis revealed that the secreted form of the recombinant protein self-assembles into trimers. Preliminary results strongly suggest that the multimeric form of HCV E2 presents is uptaken by macrophage-like THP-1 cells by receptor-mediated endocytosis.

Conclusions: The new protein design strategy which we are developing may allow the assembly of HCV E2 trimers to be tested as a vaccine candidate against HCV. Future work will aim at combining this strategy with other methods of multivalent antigen display to potentially further increase the immunogenicity of the vaccine candidates.

Resveratrol: NIR-responsive microcarrier-based polymeric delivery system to enhance its therapeutic potential towards diabetic retina cells

Roxana Pop[a,*,#], Adrian Boldianu[a,#], Mădălina Nistor[a], Maria Suciu[b], Adela Pintea[a], Monica Focsan[c], Dumitrița Rugină[a]

a) University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Calea Mănăştur 3-5, Cluj-Napoca, Romania;

b) Electron Microscopy Centre "C. Craciun", Biology and Geology Faculty, Babes-Bolyai University, 5–7 Clinicilor Street, Cluj-Napoca, Romania;

c) Nanobiophotonics and Laser Microspectroscopy Center, Interdisciplinary Research Institute in Bio-Nano-Sciences, Treboniu Laurean Street 42, Cluj-Napoca, Romania;

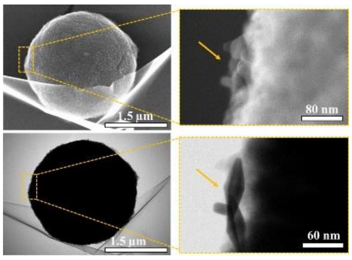
**email: roxana.pop@usamvcluj.ro # these authors contributed equally*

Introduction: Diabetic retinopathy (DR) is the leading cause of vision impairment and blindness in the world. Resveratrol (RSV) has been proposed as a potential therapeutic agent for DR owing to its anti-angiogenic and antioxidant properties [1]. However, RSV encapsulation in a delivery system is needed to improve its bioavailability and bioaccessibility.

Materials and methods: After RSV was attached to a $CaCO_3$ template, negative poly(sodium styrene sulfonate) and positive polyethylenimine polymers were alternately added using the layer-by-layer technique, whereas fluorescein isothiocyanate and gold nanoparticles were trapped between the layer. The microsystem's surface was decorated with anti-vascular endothelial growth factor (VEGF) antibodies. Morphological characterization and internalization in human retinal pigmented epithelial (RPE) D407 cells were performed using electron and fluorescence microscopy. High-performance liquid chromatography (HPLC) was used to measure the encapsulation efficiency of RSV and its release under near-infrared (NIR) laser irradiation at 785 and 808 nm. Cell viability was analyzed using the WST-1 reagent.

Results: A stable spherical shape and an average diameter of 2.5 µm were recorded through electron microscopic analysis. According to the HPLC analysis, RSV was encapsulated with an efficiency of over 90% (29.81 ± 0.04 µg/ml). Its release from the microsystem after 785 nm laser irradiation was 12.83 ± 0.21 µg/ml (43%) and after 808 nm laser irradiation was 3.89 ± 0.07 µg/ml (13,04%). Successful internalization in D407 cells was observed by fluorescence microscopy after 4–24 h of treatment with 10 microcapsules/cell. No cytotoxic effect on cells was detected. Following 808 nm irradiation, changes in cellular viability were observed [2].

Conclusions: This novel as-developed hybrid polymeric microcarrier able to release RSV by laser-induced thermoplasmonic effect could have an important translational potential for the treatment of DR.



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The endoplasmic reticulum (ER) degradation-enhancing alpha-mannosidase-like protein 3 is a host cell regulator of HBV infection

Alina Ghionescu [a,#,*], **Mihaela Uță** [a,#], **Cătălin Lazăr** [a], **Petruța Alexandru** [a], **Norica Nichita** [a]

a) Institute of Biochemistry of the Romanian Academy, Department of Viral Glycoproteins
Bucharest, Romania
**email: alinaghionescu@yahoo.com # these authors contributed equally*

Introduction: Hepatitis B virus (HBV) infection remains a serious health problem, with approximately 296 million people chronically infected worldwide. HBV is a DNA virus of the Hepadnaviridae family, that contains relaxed circular partially double-stranded DNA – rcDNA packed into a nucleocapsid enveloped by lipid bilayer embedding the small (S), medium (M), and large (L) surface glycoproteins. These proteins are synthesized in the endoplasmic reticulum (ER), where glycosylation, folding, and oligomerization, occur and are thought to induce an unfolded protein response (UPR) in HBV-infected cells. Growing evidence indicates that the ER-associated degradation (ERAD), and autophagy, two UPR pathways, are modulated by viruses in order to facilitate their replication. Indeed, non-degradative autophagy is required for nucleocapsid assembly and HBV replication. We have previously reported that HBV upregulates EDEM3 expression in hepatic cells. This ERAD lectin is involved in the degradation of the excess proteins accumulating in the ER, including the viral glycoproteins. However, the role played by EDEM3 in HBV infection is not known. The aim of this study was to investigate at the molecular level the complex interplay between UPR and the HBV life-cycle, with a focus on EDEM proteins [1,2,3].

Materials and methods: We established new HepaRG cell lines that constitutively modulate EDEM3 expression using a retroviral virus for EDEM3 overexpression and CRISPR/Cas9 technology for EDEM3 depletion. In these cells, viral infection, autophagy, and UPR markers were quantified with specific methods, including qPCR, western blotting, and ELISA.

Results: Our data indicate that HBV infection increases significantly in EDEM3-overexpressing HepaRG cells. Moreover, we observed that EDEM3 increases the secretory autophagy by microtubule-associated protein 1A/1B-light chain 3 (LC3) up-regulation and mammalian target of rapamycin (mTOR) inhibition. Additionally, EDEM3 decreases UPR markers such as protein kinase RNA-like ER kinase (PERK), inositol-requiring enzyme 1 (IRE1), and glucose-regulated protein 78 (GRP78/BiP), likely contributing to the alleviation of the ER stress during viral infection. EDEM3 depletion in HepaRG cells resulted in opposite effects, confirming the specificity of EDEM3 regulation of HBV infection.

Conclusions: Our results suggest that EDEM3 increases HBV infection by activating secretory autophagy, through modulation of the UPR signaling. Further investigation of this molecular pathway will be carried out in HBV-infected patients to potentially validate EDEMs as novel antiviral targets.

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Roles of transient receptor potential melastatin 8 (TRPM8) and epidermal growth factor receptor (EGFR) in glioblastoma

Alina-Ana-Maria Botezatu [a,*], David-Ionuț Băcioiu [a], Florentina PIciu [a], Dana Cucu [a]

a) Department of Anatomy, Aminal Physiology and Biophysics, Faculty of Biology, University of Bucharest, Splaiul Independenței 91 – 95, Bucharest 050095, Romania;
**email: botezatu.alina-ana-maria22@s.bio.unibuc.ro*

Introduction: Glioblastoma multiforme (GBM) is an extremely dangerous primary brain tumor with a low survival rate and characterized by nuclear atypia, cellular pleomorphism, mitotic activity, anaplasia, and rapid proliferation alternated with an aggressive invasion of the surrounding brain tissue [1]. Numerous functions have been linked to members of the transient receptor potential (TRP) non-selective cation channel superfamily [1]. In recent years, many reports have supported the theory that the transient receptor potential melastatin 8 (TRPM8) is a promising novel therapeutic target in cancer treatment [1]. However, the precise methods by which TRPM8 operates in glioma cells as well as its roles in carcinogenesis are still not well understood [1]. EGFRvIII, deletion of exons 2 through 7, is the most common EGFR mutation found in glioblastoma. However, the EGFRvIII mutant is classically considered low constitutively active . Also, EGFRvIII dimers can be as active as wild-type ligand-bound activated EGFR [2].

Materials and methods: We used flow cytometry to assess the influence of TRPM8 activation on cell cycle progression of U87 cells. And the wound healing technique was used to assess the roles of TRPM8 in cell migration. The experiments also follow whether the activation of EGFR influences the expression and function of TRPM8.

Results: We monitored cell migration under different conditions: activating TRPM8 by menthol and inhibition of Ca^{2+} entry using the antagonist N-(4-tertiarybutylphenyl)-4-(3-chlropyridin-2-yl)tetrahydropyrazine-1(2H)-carbox-amide (BCTC). We performed cell cycle analysis with flow cytometry to determine whether TRPM8 may change the distribution of cell cycle phases, as noticed in our previous studies for TRPA1 in pancreatic cancer cells [3]. Moreover, we quantify the influence of TRPM8 expression on cell apoptosis.

Conclusions: With this study we provide a preliminary analysis of TRPM8 expression and function in glioma cells U87 and its modulation by the EGFRvIII. TRPM8 channel may have functional implications for glioma survival, proliferation, apoptosis and local tumor invasion.

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SREB receptor family members differentially regulate protein expression and processing

Badea Rodica – Aura [a,*], Anghel Andreea Sorina [a], Stratulat Teodora [a], Tunaru Sorin [a]

a) Cell Signaling Research Group, Institute of Biochemistry of the Romanian Academy,
Splaiul Independentei 296, Bucharest, Romania
**email: Rodica.Badea@biochim.ro*

Introduction: The super-conserved receptors expressed in the brain (SREB) constitute a family of orphan G protein-coupled receptors (GPCRs) that include three members: GPR27 (SREB1), GPR85 (SREB2) and GPR173 (SREB3). They share some common features such as: high level of sequence conservation among vertebrates and predominant expression in the central nervous system. The SREB family has been postulated to play important roles in different physiologic processes and diseases, including pancreas β-cell insulin secretion and regulation, schizophrenia, autism and atherosclerosis [1]. One of the ways in which these GPCRs could influence different cellular processes is by modulating the expression and processing of proteins with important cellular functions. Our present study focuses on a comparative evaluation of the involvement of the three members of SREB family in the regulation of the expression and processing of some proteins with important roles in cell signaling.

Materials and methods: HEK293T and HeLa cells were transiently transfected with plasmids containing the cDNA of human GPR27, GPR85, GPR173, GPR75, PTGIR, β1-AR, GPR109A, a genetic probe specific for cAMP accumulation (pGloSensor), cDNA of vSrc, Lck and GRK kinases using either polyetyleneimine or Lipofectamin 2000. After 48h from transfection, cells were prepared for various investigations. The influece of of GPR27, GPR173 and GPR85 on cell signaling through G-proteins was determined by a luminiscence-based assay using pGloSensor (Promega) genetic probe. The impact of SREB family members on protein expression levels of some other GPCRs (GPR75, PTGIR, β1-AR) and kinases (vSrc, Lck, GRKs) was assessed by western blotting, while their effect on gene transcription was performed by qPCR.

Results: Functional assays demonstrated an inhibitory effect of GPR27 and GPR173 on forskolin-induced cAMP accumulation in HEK293T and HeLa cells. Interestingly, these effects were insensitive to a Gi-specific blocker, pertussis toxin (PTX). Further on, the data obtained from immuno-blotting experiments have shown that both GPR27 and GPR173 specifically inhibit the expression of some co-expressed proteins, such as GPR75, β1-adrenergic receptor (β1-AR) and prostacyclin receptor (PTGIR), protein tyrosine kinases (Src) and protein serin/threonin kinases (GRKs), whereas the expression of a Src-related kinase such as Lck was not affected. GPR85 did not have inhibitory effects on the aforementioned proteins. The overall results sugest that GPR27 and GPR173 play specific inhibitory effects on the expression of certain proteins while the GPR85 seems to not affect the protein expression. Further studies are required to clarify the role of SREB family receptors in cellular physiology.

Conclusions: GPR27 and GPR173 modulate various physiological processes by influencing the expression of some important proteins in cell signaling (GPCR, G protein subunits, kinases) by an yet unknown mechanism.

Acknowledgement: This work was funded from "EEA-RO-NO-2018-0535 - New Generation of Drug Targets for Schizophrenia (NEXTDRUG)".

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Data mining and protein engineering for aromatic ammonia-lyases with enhanced activity towards challenging substrates

Gyopárka Ágoston [*], **Krisztina Boros, Raluca Bianca Tomoiagă, Dr. László-Csaba Bencze**

Enzymology and Applied Biocatalysis Research Center, Faculty of Chemistry and Chemical Engineering,
Babeș-Bolyai University, Arany János Str. 11, RO-400028 Cluj-Napoca, Romania
**email: gyoparka.agoston@stud.ubbcluj.ro*

Introduction: Phenylalanine ammonia-lyases (PALs) have emerged as a powerful tool in the production of optically pure L-phenylalanines, providing a greener approach with high conversion rates. Together with histidine and tyrosine ammonia-lyases (HALs and TALs), these enzymes harbour the catalytically indispensable 3,5-dihydro-5-methylidene-4H-imidazole-4-one (MIO) prosthetic group, thereby constituting the MIO enzyme family. A major limitation of PALs is their low activity towards electron donating poly-substituted substrates, a problem which so far could not be solved by the means of protein engineering [1]. Recently, a new aromatic ammonia-lyase (AAL) was discovered, derived from *Loktanella atrilutea* (*La*AAL) showing high activity in the production of 3,4-dimethoxy-L-phenylalanine (Fig. 1), prominent as an intermediate in the synthesis of the anti-Parkinson drug L-DOPA [2]. The focus of this study is the exploration of the natural role and substrate domain of *La*AAL.

Materials and methods: Employing the Basic Local Alignment Search Tool (BLAST) homologues of *La*AAL were selected, with the highest rates of coverage and identity values above 60%. The evolutionary lineage relationships between these homologues and known members of the MIO enzyme family were mapped through phylogenetic tree analysis, using the Molecular Evolutionary Genetics Analysis (MEGA11) program. MUltiple Sequence Comparison by Log-Expectation (MUSCLE) was applied comparing the catalytic sites of the homologues and point mutations were intorduced to *La*AAL using megaprimer based polymerase chain reactions (PCR). Three mutant enzymes were transformed and expressed in *E. coli* competent cells. Their conversion-based activities were monitored with RP-HPLC and compared to the native enzyme within the ammonia addition and elimination reaction.

Results: While, the MIO prostetic group is present in all the selected AAL homologues, their catalytic site is unique compared to other characterized MIO-family members. On the generated phylogenetic diagram, the examined AALs form a distinct clade, having common ancestors with PALs and TALs of bacterial origin. Between the identified AALs, there are well-conserved residues specific to MIO-family members, but a vast diversity was observed amongst the residues defining the substrate selectivity. To assess how these distinct residues affect the activity, several mutations that interchange the catalytic sites of the AALs and of the known PALs were performed, testing the activity of the variant library towards a defined set of differently substitued substrates.

Conclusions: A phylogenetical analysis was performed on the novel aromatic ammonia-lyase from *Loktanella atrilutea* and its homologues together with known MIO-family representatives. The mutational strategy implemented by interchanging the catalytic sites of the different PAL homologues, provides knowledge for developing protein engineered PALs, capable of transforming lignin-derived monomers, including syringic acid and ferulic acid.

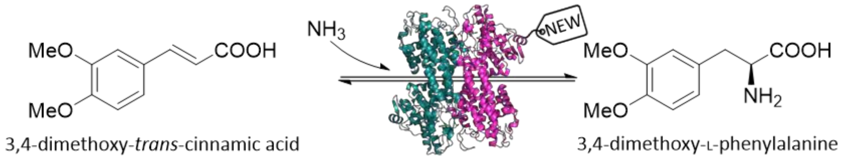

Fig. 1: Ammonia addition reaction of 3,4-dimethoxy-*trans*-cinnamic acid catalysed by *La*AAL.

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Fluorescence imaging in real-time employed to visualize the presence of anthocyanins complexed with diphenylboric acid 2-aminoethyl inside B16-F10 melanoma cells

Madalina Nistor [a,*], **Monica Focsan** [b], **Luiza Gaina** [c], **Mihai Cenariu** [a], **Adela Pintea** [a], **Carmen Socaciu** [a], **Dumitrita Rugina** [a]

a) University of Agricultural Sciences and Veterinary Medicine, Manastur Street 3-5, Cluj-Napoca, 400372, Romania
b) Nanobiophotonics and Laser Microspectroscopy Center, Interdisciplinary Research Institute in Bio-Nano-Sciences, Treboniu Laurean Street 42, Cluj-Napoca, 400271, Romania
c) Babes-Bolyai University, Faculty of Chemistry and Chemical Engineering, Arany Janos Street 11, Cluj-Napoca, 400028, Romania
**email: nistor.madalina@usamvcluj.ro*

Introduction: Anthocyanins (AN) are natural antioxidant compounds regularly consumed by humans, that possess remarkable potential for therapeutic use when applied directly to melanoma pathology. However, the development of an effective therapy is still underway due to the limited understanding of how AN molecules behave within cells, once they are absorbed. A major obstacle in this endeavor is the requirement for an imaging technique that can accurately detect and locate AN molecules within cells. To address this challenge, a non-fluorescent reagent called diphenylboric acid 2-aminoethyl (DPBA) was utilized in this study to successfully create fluorescent complexes with AN, offering a solution for their intracellular detection and localization.

Materials and methods: The AN used in this study consisted of cyanidin aglycon as standard molecule (CY) and glycosylated compounds extracted, purified from chokeberry fruits (AE) and quantified by high-performance liquid chromatography with photodiode array detector (HPLC/PDA). Observation of CY/AN inside B16-F10 melanoma cells was done using flow cytometry and fluorescence microscopy. To enhance their fluorescence detectability, AN/CY were conjugated with DPBA, and formed fluorescent complexes, as proven by nuclear magnetic resonance (NMR) analysis. Additionally, their impact on cellular viability was also analysed through the colorimetric WST-1 assay.

Results: The results showed that AN form fluorescent complexes with DPBA within the melanoma cells, resulting in an enhanced fluorescent signal. After 24 hours, 62.9% of the B16-F10 cells treated with AE and 64.8% of cells treated with CY, emitted fluorescence. Fluorescence microscopy imaging and flow cytometry analysis validated the cellular uptake of the complex. Also, the viability test showed that AN had cytotoxic effect on melanoma cells, on a dose-dependent manner, while DPBA showed no impact on viability, at the concentrations used in this study.

Conclusions: This study demonstrated that DPBA can be effectively utilized as a sensitive and non-invasive method for imaging AN intracellularly. The success of this approach opens up new possibilities for its application in various fields, such as drug development and the study of metabolism-associated mechanisms. The findings suggest that DPBA has the potential to significantly enhance our understanding of AN behavior within cells and can have broad implications for further research and applications in related areas.

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Integrons and HGT – How do integrons affect HGT in multidrug resistant Gram-negative ESKAPE pathogens

Matei-Ștefan Dobrescu [a,b,c*], Dan Alexandru Țoc [d], Anca Butiuc-Keula [b]

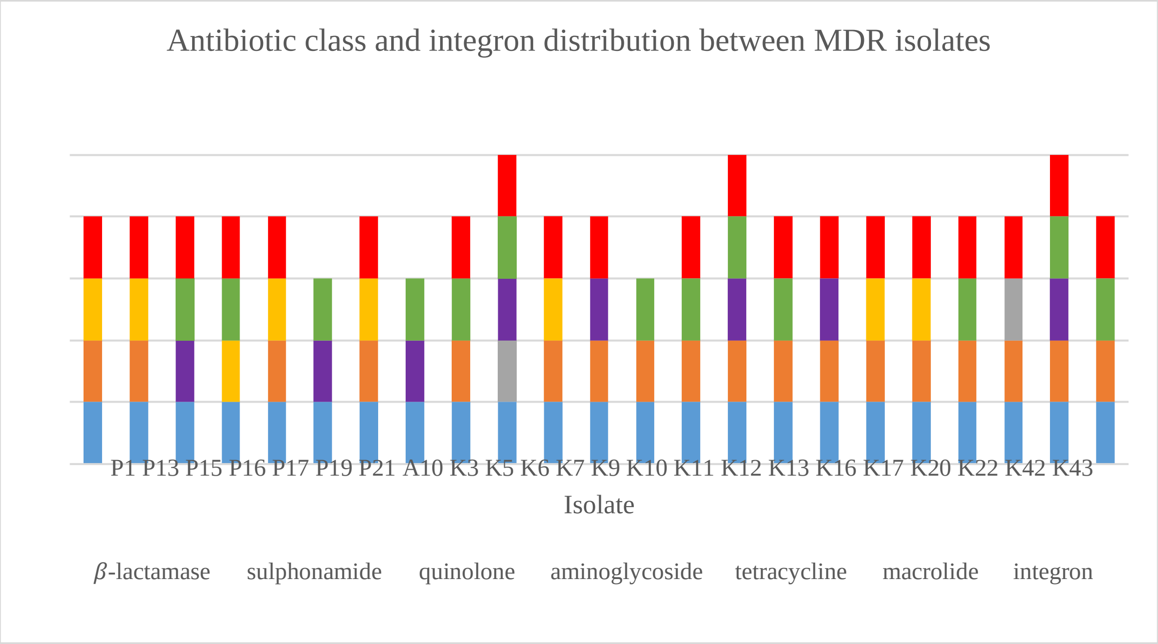
a) Department of Molecular Biology and Biotechnology, Faculty of Biology and Geology,
Babeș-Bolyai University, 1 M. Kogalniceanu Street, 400084 Cluj-Napoca, Romania
b) Centre for Systems Biology, Biodiversity and Bioresources, Babeș-Bolyai University, 5–7 Clinicilor Street, 400006 Cluj-Napoca, Romania
c) Doctoral School of Integrative Biology, Babeș- Bolyai University, 44 Republicii street, 400015 Cluj-Napoca, Romania; d) Department of Microbiology, Iuliu Hatieganu University of Medicine and Pharmacy, 8 Victor Babeș Street, 400000 Cluj-Napoca, Romania
**email: matei.dobrescu@ubbcluj.ro*

Introduction: Bacterial resistance to antibiotics is a public health problem due to the stress these substances place on bacteria, causing them to acquire antibiotic resistance genes (ARGs). Horizontal gene transfer (HGT) allows bacteria to obtain new genetic material from outside their own species or from individuals within the same species. Through HGT, bacteria can acquire new genes that confer characteristics that can be useful and help them to perpetuate the species in a given environment. ARGs can be obtained through HGT, one of the main ways these genes being acquired through mobile genetic elements (MGE), especially integrons. Through the acquisition of ARGs, some bacteria can become multidrug resistant (MDR). Over the years, a group of such bacteria have gained notoriety, being placed in a group named ESKAPE pathogens. In this group, we can find bacteria such as Enterobacter spp., *Klebsiella pneumoniae*, Acinetobacter baumannii and Pseudomonas aeruginosa.

Materials and methods: The clinical isolates used were isolated from patients from the Cluj County Emergency Hospital. We have analyzed 95 isolates from 4 different bacterial species: *Enterobacter* spp. (E), *Klebsiella pneumoniae* (K), *Acinetobacter baumannii* (A) and *Pseudomonas aeruginosa* (P). 40 ARGs and 3 integrase genes (intI, intII, intIII) were tested by using PCR and electrophoresis to identify which ARGs and which integron classes are present. The data was analyzed using Microsoft Excel and statistical tests (ANOVA; Mann–Whitney U and Kruskal–Wallis) were done using the R Studio software.

Results: Out of all 40 ARGs, we have only identified 20. These 20 ARGs are shared between 6 antibiotic classes: β-lactamases, macrolides, tetracyclines, sulphonamides, aminoglycosides and quinolones. Out of all 95 isolates, 23 of them can be considered MDR, meaning they are resistant to at least 3 antibiotic classes. Out of all the isolates, we have identified 41 isolates having integrons. Regarding the MDR isolates, only 19 out of 23 have integrons. Regarding the integrons, the most present class was class I (36 isolates). Only 6 isolates have class II integrons and none have class III. We have analyzed the impact of integrons over each antibiotic class. In the case of suphonamides, 25 out of 29 isolates present integrons (86,2%). For macrolides, 15 out of 27 present integrons (55,5%). For aminoglycosides, 8 out of 10 isolates present integrons (80%). For tetracyclines, 6 out of 23 present integrons (26%). For β-lactamases, 39 out of 77 isolates present integrons (50,64%). When looking at quinolones, there is only one isolate that has resistance to them and it also contains integrons. When analyzing the presence of integrons in MDR isolates, 20 out of 23 have only class I integrons (86,95%). After statistical analysis, we can observe a possible correlation between the dissemination of sulphonamides and the presence of integrons. Regarding the other classes, there isn't a significant correlation. However, there might be a correlation between integrons and the dissemination of macrolides in the case of Enterobacter spp as only the isolates with integrons present resistance to macrolides. When looking at the MDR isolates, there is a significant correlation between the presence of integrons and the MDR phenomena.

Conclusions: Integrons can facilitate the movement of certain antibiotic classes such as sulphonamides and macrolides. We can confirm that some antibiotic classes might move independently from integrons, such as β-lactamases which are known to move with the aid of plasmids. The MDR phenomena might be affected by integrons, helping the spread of ARGs to bacteria who are already resistant to multiple antibiotic classes.



Unchanged amount, localization and activity of glucocorticoid receptor in combination therapy - inhaled nitric oxide and intravenous hydrocortisone in porcine endotoxemia model.

Michał Płóciennik [a*], Liliana Kiczak [a], Urszula Pasławska [b,c], Waldemar Gozdzik [d], Barbara Adamik [d], Marzena Zielinska [d], Stanisław Zieliński [d], Kacper Nowak [c], Jacek Bania [e], Marcin Nowak [f], Robert Pasławski [b], Claes Frostell [g]

a) Department of Biochemistry and Molecular Biology, Faculty of Veterinary Medicine, Wroclaw University of Environmental and Life Sciences, Wroclaw, Poland
b) Veterinary Center, Nicolaus Copernicus University in Torun, Torun, Poland
c) Department of Internal Diseases and Clinic of Diseases of Horses, Dogs and Cats, Faculty of Veterinary Medicine, Wroclaw University of Environmental and Life Sciences, Wroclaw, Poland
d) Clinical Department of Anesthesiology and Intensive Therapy, Wroclaw Medical University, Wroclaw, Poland
e) Department of Food Hygiene and Consumer Health Protection, Faculty of Veterinary Medicine, Wroclaw University of Environmental and Life Sciences, Wroclaw, Poland
f) Department of Pathology, Faculty of Veterinary Medicine, Wroclaw University of Environmental and Life Sciences, Wroclaw, Poland
g) Department of Anesthesia and Intensive Care, Karolinska Institutet, Danderyd Hospital, Stockholm, Sweden
**email:* michal.plociennik@upwr.edu.pl

Introduction: Despite modern intensive care the hospital mortality in sepsis still varies between 30–45% [1]. Multiple preclinical studies have proven the central role of endogenous glucocorticoids tolerance against sepsis by counteracting several of the sepsis characteristics i.a. excessive inflammation [2]. Therefore, glucocorticoids (GCs) have been tested as an adjunctive therapy in sepsis and septic shock in different randomized clinical trials. Nonetheless, these studies produced conflicting results [2]. Glucocorticoid resistance (inadequate response of the glucocorticoid receptor, GR) is a well-known manifestation in sepsis and may contribute to the failure of GCs to improve sepsis patients [2]. On the other hand, a combination therapy - simultaneous administration of both a corticosteroid and an inhaled nitric oxide (iNO) in a porcine endotoxemia model (infusion of lipopolysaccharide, LPS) attenuated the inflammatory response and almost preserved or restored normal histology of both lung and systemic organs [3]. Thus, we decided to evaluate the influence of this combination therapy on amount and localization of GR in lung, kidney and liver, using two complementary methods – immunohistochemistry and Western blotting (after protein subfractionation) as well as GR activity (expression of genes controlled by GR).

Materials and methods: A shock-like condition was established in 23 animals by continuous infusion of *E. coli* LPS for 10 hours. Then the animals were observed for 10 hours. 12 pigs received iNO and hydrocortisone (iNO treatment started 3 hours after the initial LPS infusion and continued until experiment end). 11 pigs were controls (with standard treatment). Cytoplasmic and nuclear protein fractions were extracted from lungs, liver, and kidney for Western blot analysis of GR. GR localization in porcine lungs, liver, and kidneys was analyzed using immunohistochemistry (IHC). Expression of proinflammatory genes (IL-1β, TNF-α, IL-6, TGF-β) was measured by real-time PCR.

Results: Western blotting demonstrated that amount of GR in cytoplasmic and nuclear protein fractions in lungs, kidneys and livers remained on similair levels in **pigs treated with iNO and** hydrocortisone and in controls. The IHC image digital analysis with Image J program revealed that the cytoplasmic and nuclear staining pattern was comparable in both analyzed groups. **Expression of proinflammatory genes, controlled by GR, remained on similar level in all animals.**

Conclusions: Animals treated with the combination of iNO and corticosteroid and controls represented similar amounts, localization, and activity of GR in pulmonary, hepatic and renal tissue. Our findings suggest that the combination therapy with GCs and iNO might prevent **glucocorticoid resistance** in sepsis therapy.

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Altered expression of microRNA-195 might contribute to a severe outcome in SARS-CoV-2 infection

Moatăr Alexandra-Ioana [a *], **Chiș Aimee Rodica** [a], **Nitusca Diana** [a], **Marian Cătălin** [a], **Oancea Cristian** [b], **Sîrbu Ioan-Ovidiu** [a]

a) Department of Biochemistry, University of Medicine and Pharmacy"Victor Babeș", E.Murgu Square no.2, Timișoara, Romania;
b) Department of Pneumology, University of Medicine and Pharmacy"Victor Babeș", E.Murgu Square no.2, Timișoara, Romania;
**email: moatar.alexandra@umft.ro*

Introduction: While the infections with SARS-CoV-2 have reached a "steady state" globaly, there is still a dire need to understand the molecular mechanisms that contribute to the long term effects of COVID-19[i]. Human microRNAs, as important modulators of gene expression, are frequently dysregulated in response to viral infection [ii]. MicroRNA-195, in particular, was found to interact 'in silico' with the N gene of SARS-CoV-2 genome, however, it is not clear how this molecule associate to and impact the progression of COVID-19 disease [iii]. Here, we quantified the plasma levels of microRNA-195 in COVID-19 patients and evaluated its association with progressive stages of the disease.

Materials and methods: We used qRT-PCR to profile the expression of microRNA-195, in plasma samples of COVID-19 patients admitted to the Clinical Hospital of Infectious Diseases and Pneumophysiology, Timisoara. We used mirWalk 3.0 and STRING algorhitms to evaluate the biological impact of microRNA-195 on lung, heart, lymph nodes, liver and kidneys transcriptomes. The statistical significance of changes in microRNA-195 expression was calculated using Kruskal Wallis test with Dunn's multiple corrections. Correlation analyses were performed using the two-tailed Spearman test (continuous variables), Point Bisserial test (continuous vs. binary variables) and Phi test (binary variables). Receiver operating characteristics (ROC) analyses were performed with the standard parameters in Prism 9, using Wilson/Brown method for confidence interval calculation. All statistical analyses were two tailed and the threshold of statistical significance was set at 0.05.

Results: Plasma microRNA-195 is strongly downregulated in patients infected with SARS-CoV-2 virus compared to controls, an effect dependent on the severity of the SARS-CoV-2 infection. ROC curve analyses showed that plasma microRNA-195 has a high discriminative power (AUC>0.9, p<0.0001) for severe COVID-19 cases vs. controls and COVID-19 mild cases. Results from the bioinformatic analysis showed that microRNA-195 significantly impacts the mitochondrial respiration in cardiac muscle. Furthermore, our correlation analysis revealed that plasma microRNA-195 is negatively correlated with exitus (p=0.008), D-dimers (p=0.041) and age (p=0.018).

Conclusions: Plasma microRNA-195 downregulation is strongly associated with early signs of COVID-19 disease severity. Our work points towards a previously unknown role of microRNA-195 host response to SARS-CoV-2 infection cases.

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NPC1 regulates specific cargo traffic to lysosome related organelles and melanosome biogenesis

Rus Adriana Alina[a *], **Ioana Militaru**[a], **Ioana Popa**[a], **Ştefana Petrescu**[a]

a) Department of Molecular Cell Biology, Institute of Biochemistry, Splaiul Independenței 296, 060031, Bucharest, Romania
**email: rus.alinaa@yahoo.com*

Introduction: Niemann-Pick type C1 protein (NPC1) is a multi-spanning protein of lysosomes membrane that enables cholesterol and lipids transport. Mutations in the NPC1 protein can lead to Niemann-Pick type C1 disease, a lysosomal storage disorder, characterized by the accumulation of glycosphingolipids and cholesterol within lysosomes. NPC1 protein plays a major role in the endolysosomal pathway, therefore we have investigated its role in a lysosome-related organelle, the melanosome [1,2].

Materials and methods: We used CRISPR/CAS9 engineering method to generate a new cell line lacking NPC1 protein. Other methods such as Western blot, immunofluorescence staining, subcellular fractionation and flow cytometry were used to characterise the perturbed processes in cell lines deficient in NPC1 protein.

Results: We have generated a knock-out NPC1 melanoma cell line, MNT-1 NPC1-KO (NPC1-KO), and we found that the cellular phenotype is associated with a decreased pigmentation accompanied by low expression of the melanogenic enzyme tyrosinase. We investigated the pathway of tyrosinase and melanosome biogenesis. In the absence of NPC1 protein not just the expression of tyrosinase, but also the processing and the localization were affected. In contrast with the decrease in tyrosinase protein expression, we observed a significant intracellular accumulation of mature PMEL17, the structural protein of melanosomes. Therefore, in NPC1-KO cell line the melanosomal matrix generation is unable to lead to the maturation of melanosomes causing them to remain immature with a subcellular localization adjacent to the plasma membrane, in contrast to the dendritic melanosomal localization in wild type cells.

Conclusions: In conclusion, these findings suggest that NPC1 is involved in tyrosinase transport from the TGN to melanosomes and melanosome maturation, indicating a novel function for the NPC1 protein.

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GPR27, insulin secretion regulator and beyond

Sorina-Andreea Anghel [a,*], **Cosmin Trif** [a], **Rodica-Aura Badea** [a], **Aura-Elena Ionescu** [a],
Teodora Stratulat [a], **Cristiana Triță** [a], **Sorin Tunaru** [a], **Ștefana M. Petrescu** [b]

a) Cell Signaling Research Group, Institute of Biochemistry of the Romanian Academy,
Splaiul Independenței 296, Bucharest, Romania
b) Department of Molecular Cell Biology, Institute of Biochemistry of the Romanian Academy,
Splaiul Independenței 296, Bucharest, Romania
**email: anghelsandreea22@gmail.com*

Introduction: G-protein coupled receptors (GPCRs) are ubiquitously expressed in the human body and play essential roles in an array of physiological processes, including glucose homeostasis. Orphan GPCRs are receptors that lack endogenous ligands and biological functions, thus being potential novel targets for drug discovery. GPR27 is an orphan receptor belonging to the SREB family (Super-conserved Receptors Expressed in Brain) together with GPR85 and GPR173. Previously, GPR27 was linked with increased insulin production [1]. We pursued to define GPR27 signaling in insulinoma cell line (INS1E) and beyond, in order to establish its role in insulin secretion.

Materials and methods: Secondary messengers were quantified by using cytosolic biosensors transiently expressed together with GPR27 in HEK293T cells or in INS1E cell line. cAMP levels were determined using pGlo22F system (Promega), while G5A (GFP-aequorin fusion protein) detected changes of intracellular calcium. The G-proteins activity was determined using TRUPATH biosensors [2]. Expression of GPR27 in INS1E cells was performed by qPCR. Insulin secretion was measured by HTRF (Cisbio).

Results: We demonstrated that GPR27 is expressed in INS1E cells. Firstly, we sought to characterize GPR27 signaling in a heterologous expression system. In HEK293T cell line, GPR27 induced a decrease of cAMP in an agonist-independent manner suggesting a constitutive activity mediated by Gαi/o proteins. However, pertussis toxin (PTX) or (OZITX), inhibitors of Gαi/o proteins, did not affect the observed decline in cAMP levels. Moreover, GPR27 did not recruit any Gα protein as assessed by TRUPATH method. Next, by using a functional approach we demonstrated that GPR27 blocks the activity of free-fatty acids receptors (FFAR1, FFAR4) and muscarinic acetylcholine receptors (M1, M3, M5), well-known Gαq coupled receptors expressed in beta-cells. Furthermore, knockdown of GPR27 in INS1E cells by siRNA strategy resulted in elevated intracellular calcium signaling and augmented glucose-stimulated insulin secretion.

Conclusions: GPR27 exerts an inhibitory effect in an agonist-independent manner affecting the activity of free-fatty acids receptors (FFAR1, FFAR4) and muscarinic acetylcholine receptors (M1, M3, M5) via a G-protein independent pathway. Likewise, the SREB member seem to negatively regulate insulin secretion.

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GPR27 inhibits Hepatitis B Virus internalisation and replication

Stefania Buzatoiu[*], **Sorin Tunaru, Norica Nichita**

Institute of Biochemistry of the Romanian Academy, Splaiul Independenței 296,
București 06003, Romania
**email: stefaniabuzatoiu@gmail.com*

Introduction: Hepatitis B Virus (HBV) is the cause of 800.000 annual deaths out of 296 million of chronically-infected patients worldwide, for whom no curative treatment is currently available. Previous studies have indicated that the HBV life-cycle and the host cell metabolism are tightly interdependent. Understanding the molecular details of these interactions may reveal novel antiviral targets and therapeutic strategies. G protein coupled receptors (GPCR) constitute the largest superfamily of human membrane receptors involved in signal transductions through activation of intracellular G proteins. GPCRs regulate various physiological processes and are one of the most important group of pharmacological targets. We have recently shown that a member of this family, GPR27 is expressed in hepatic cells . Our hypothesis is that GPR27 may regulate HBV infection through activation or inhibition of host cell pathways involved in the viral life-cycle.

Materials and methods: Transient overexpression of GPR27 in hepatoma cell lines. HepG2-NTCP cells were treated with 0,25 µg pcDNA-GPR27/ pcDNA plasmids for 24; GPR27 overexpression was further confirmed by western blot and immunorescence assays. **In vitro HBV infection**. HepG2 NTCP cells were incubated with HBV at 300 MOI 24h post GPR27 overexpression and harvested after 24h or 7 days, respectively. **Quantification of HBV antigens secretion by ELISA**. The amount of HBV-SVPs released was quantified using the Monolisa HBsAg Ultra Kit according to the protocol recommended by the manufacturer. Secretion of the HBeAg was monitored by using the Monolisa HBe Ag-Ab PLUS.**Viral DNA ectraction** was performed using Phenol/Chloroform/Isoamyl Alcohol Extraction protocol and qPCR reaction products were amplified using HBV primers.

Results: Our results suggest that GPR27 overexpression could inhibit HBV entry and replication, leading to a decreased infection rate.

Conclusions: In this study, we have established that GPR27 is a novel host factor involved in HBV infection. Future research will aim to determine the molecular mechanism of this inhibition and identify GPR27-targetting drugs with antiviral properties.

Analytical differences between hemoglobin peroxidase and enzyme-based peroxidase activity in tissue lysates

Ștefan-Mihai DRĂGAN [1b,*], **Vlad-Alexandru TOMA**[1 a,b,c]

a) Department of Molecular Biology and Biotechnology, Faculty of Biology and Geology, Babeș-Bolyai University, Clinicilor Street No. 4-7, 400006, Cluj-Napoca, Romania;
b) Institute of Biological Research, Republicii Street No. 48, 400016, Cluj-Napoca, the branch of NIRDBS Bucharest, Romania),
c) „Maya & Nicolae Simionescu" Fellow, Romanian Society for Cell Biology, Republicii Street No. 48, 400016, Cluj-Napoca, Romania

**email: stefan.dragan@stud.ubbcluj.ro*

Introduction: The peroxidase-like activity of hemoglobin has been well described in the literature over the years, due to its hem prosthetic group which can catalyze the breakdown of peroxide into H_2O and ROS (1). Stroma-free hemoglobin can arise due to erythrocyte's plasma membrane destruction, resulting in hemoglobin release, or from perfusion of hemoglobin-based blood substitutes (2). Since naturally when measuring the peroxide activity in a tissue, it is over-amplified in value by the other peroxidase activities as such of Hb but also other heme-containing proteins like cytoglobin, neuroglobin, and androglobin, the need for this discrimination becomes clear, to precisely measure the internal, intracellular peroxidase activity, and accounting for the external sources of influence on the cellular oxidative stress(3).

Materials and methods: Using the UV-Vis spectrophotometer, we managed to provide a method that relies on the oxidation state of σ-dianisidine (method 1) to register a color change proportional to the quantity of stroma-free Hb present in the sample. Other two methods are proposed to validate the method based on the oxidation state of σ-dianisidine. The ABTS colorimetric assay (method 2), in which the color change to the green of the solution can be measured at 410 nm will be proportional to peroxidase activity in the solution. The third approach is based on the reaction between the hydroxyl radicals generated by the Fenton reaction and compounds, that upon hydroxylation will produce a fluorescent signal that can be measured accordingly such as sodium terephthalate or coumarin, thus allowing the precise assessment of peroxidase activity related to Hb vs whole peroxidase activity in the tissue homogenate (method 3).

Results: We found that the additional peroxidase activity of Hb can be distinguished from the peroxidase activity of true peroxidase and can be easily calculated and subtracted for a more precise estimation of oxidative stress reduction in the sample. Accordingly, the hemoglobin concentration can be calculated using Lambert-Beer law, $C\ (\mu M) = A_{410\ nm} / \varepsilon$, ε having a value of $0.46\ \mu M^{-1}\ cm^{-1}$. To obtain the true values of native peroxidase present in the tissue, we sought to create a standard curve of peroxidase and a mathematical formula for defining the additional peroxidase activity of hemoglobin in the tissue lysates. The formula $C\ (U/mL) = \frac{\Delta abs + 0{,}1203}{0{,}06295}$, gives us the exact value in units of true peroxidase, and the possibilities for this peroxidase activity to be subtracted from the total peroxidase activity in any given tissue lysates.

Conclusions: When measuring the peroxidase activity in a tissue lysates sample, the presence of Hb is a *bona fide* factor, which must be considered to properly estimate the oxidative stress in the sample, following a drug therapy applied. Usually, this pseudo-peroxidase activity is oversight, but nonetheless, it is present, and it should be considered. This activity is present in certain conditions, mainly pathological, in which both components are present, stroma-free Hb and hydrogen peroxide. Methods were comparable and the procedure based on the oxidation state of σ-dianisidine was the most reliable protocol.

Future perspectives will take into account two other sensitive methods for determining the peroxidase activity of Hb in tissue homogenates such as RAMAN and EPR methods for heme detection and correlation to peroxidase values.

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POSTERS: Molecular evolution and phylogenetics

A highly syntenic nic-genes cluster is present in several related bacterial strains

Amada El-Sabeh [a], Andreea-Mihaela Mlesnita [a], Iustin-Tiberius Munteanu [a], Iasmina Honceriu [a], Fakhri Kallabi [a,b], Razvan-Stefan Boiangiu [a], Marius Mihasan [a*]

a) BioActive Research Group, Faculty of Biology, Alexandru Ioan-Cuza University of Iasi, Blvd Carol I no. 20A, Iasi, Romania;
b) Laboratory of Human Molecular Genetics, Faculty of Medicine of Sfax, University of Sfax, Tunisia;
**email: marius.mihasan@uaic.ro*

Introduction: The pyridine-pathway for nicotine degradation present in *Paenarthrobacter nicotinovorans* ATCC 49919 can serve as a model for studying the molecular evolution of catabolic pathways. In this current study, the newly released genome of the strain [1] was used as a reference and an extensive comparative genomics study was performed on a total of 65 related genomes.

Materials and methods: All pairwise comparisons among the set of genomes were conducted using GBDP available in TYGS, digital DNA-DNA hybridization (dDDH) values and confidence intervals were calculated using GGDC 3.0 and average nucleotide identity (ANI) values were calculated using the OrthoANI algorithm. Species clustering was done using a 70% dDDH cutoff. Comparative analysis of *nic*-genes cluster was performed with progressive Mauve using the HOXD scoring matrix.

Results: Five *Arthrobacter* strains showing both dDDH and ANI values over the species threshold when compared to *P. nicotinovorans* ATCC 49199 were identified. Five plasmids and two contigs belonging to *Arthrobacter* and *Paenarthrobacter* strains were shown to be virtually identical with the pAO1 plasmid of *Paenarthrobacter nicotinovorans* ATCC 49919. Moreover, a highly syntenic *nic*-genes cluster was identified on four plasmids, one contig and four chromosomes. The *nic*-genes cluster contains two major locally-colinear blocks (LCBs) that apparently form a putative catabolic transposon.

Conclusions: Although the origins of the *nic*-genes cluster, the putative transposon and the pAO1 megaplasmid still elude us, we hypothesise here that the ATCC 49919 strain most probably evolved from Paenarthrobacter sp. YJN-D or a very closely related strain by acquiring the pAO1 megaplasmid and the nicotine degradation pathway.

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The intricate relationship between the CRISPR-Cas system and antibiotic-resistance genes in *Klebsiella pneumoniae*

Dumitrana Iordache [a,c*], **Matei Dobrescu** [a], **Andreea Fita** [b], **Anca Butiuc-Keul** [b,c]

a) Doctoral School of Integrative Biology, Babeș-Bolyai University, 44 Republicii street, 400015 Cluj-Napoca, Romania;
b) Department of Molecular Biology and Biotechnology, Faculty of Biology and Geology, Babeș-Bolyai University, 1 M. Kogalniceanu Street, 400084 Cluj-Napoca, Romania
c) Centre for Systems Biology, Biodiversity and Bioresources, Babeș-Bolyai University, 5–7 Clinicilor Street,400006 Cluj-Napoca, Romania
**email: dumitrana.iordache@ubbcluj.ro*

Introduction: *Klebsiella pneumoniae* has emerged as the predominant pathogen responsible for nosocomial infections and exhibits significant levels of antibiotic resistance. Bacteria employ the CRISPR-Cas system to restrict the intake of foreign genetic material, effectively maintaining an equilibrium between acquiring advantageous traits through horizontal gene transfer (HGT) and safeguarding against bacteriophage infections. Research has revealed the presence of Type I-E and subtype I-E∗ CRISPR-Cas systems within *K. pneumoniae* strains [1]. This study aimed to assess the implication of the CRISPR-Cas system in the acquisition of antimicrobial resistance genes (ARG) by *K. pneumoniae* genomes.

Materials and methods: The current study employed a comprehensive approach to investigate *K. pneumoniae* genomes. A total of 100 complete genomes with CRISPR-Cas type I-E, 100 genomes with CRISPR-Cas type I-E*, and 100 genomes lacking CRISPR-Cas were downloaded from the CRISPRCasdb database. Subsequently, the CRISPR spacers within these genomes were scrutinized for potential matches against plasmids (RefSeq-Plasmid), viruses (IMGVR, RefSeq-Phage, RefSeq-Viral, and PHAST), as well as ARGs (Comprehensive Antibiotic Resistance Database) and integrons (INTEGRALL database), using the CRISPRTarget program and a custom Python script. Moreover, the presence of ARGs within the sequenced genomes was examined using the same script. The Kolmogorov-Smirnov test was conducted to evaluate normal distribution, followed by the Kruskal-Wallis test to assess parametric differences. Additionally, chi-square tests were utilized to investigate associations between counts or frequencies. For all statistical analyses, significance was determined at a p-value threshold of less than 0.05.

Results: In K. pneumoniae, the type I-E system has a single locus downstream of the Cas cluster, whereas the I-E* system typically exhibits two loci positioned both upstream and downstream of the Cas cluster. Notably, the number of spacers within the I-E system is generally higher than the combined spacers within the two I-E* CRISPR loci. Furthermore, the I-E* subtype is also distinguished from the I-E type by the sequence of the direct repeat. The spacers present in each locus are distinct, with no shared spacers observed among the three loci; however, instances of plasmids and viruses exhibiting a perfect match between spacers from both systems have been identified. The analysis demonstrated that most identified spacers originated from viral sources. While no spacers were found to correspond to antibiotic resistance genes (ARGs) or integrase sequences, certain spacers did target plasmids that may carry ARGs. Interestingly, genomes harbouring the I-E* system exhibited a significantly lower presence of ARGs compared to genomes possessing the I-E system ($p=0.0239$). Nevertheless, when compared to genomes lacking the CRISPR-Cas system, while a difference was observed, it was not statistically significant ($p=0.0587$).

Conclusions: These findings indicate that the CRISPR-Cas system in *K. pneumoniae* may primarily serve as a defence mechanism against bacteriophage infections rather than directly targeting ARGs or integrase sequences. However, the system could still indirectly impact the dissemination of ARGs by targeting plasmids carrying these genes. Although the I-E system does not impede the acquisition of ARGs, the I-E* system may possess a certain level of activity in this regard.

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POSTERS: Omics-based developments in bio-medical sciences

Ion mobility mass spectrometry of human cerebellum gangliosides

Maria-Roxana Biricioiu [a,b,*], **Mirela Sarbu** [a], **Raluca Ica** [a], **Alina D. Zamfir** [a,c]

a) National Institute for Research and Development in Electrochemistry and Condensed Matter, Timisoara, Romania;
b) Faculty of Physics, West University of Timisoara, Timisoara, Romania;
c) Department of Technical and Natural Sciences, "Aurel Vlaicu" University of Arad, Arad, Romania
**email: maria.biricioiu99@e-uvt.ro*

Introduction: Cerebellum represents a highly specialized region of the brain encompassing approximately 80% of the total brain neurons and regular arrays of neuronal units involved in motor control, learning, cognitive and emotional functions [1, 2]. In recent years, human cerebellum started to be investigated at the molecular level, in order to correlate its functions with the expression of various biomolecules, especially gangliosides, sialylated glycosphingolipids highly expressed in the central nervous system. Among the bioanalytical methods employed so far in the analysis of cerebellar biomolecules, mass spectrometry (MS) provided the most comprehensive information [3].

Materials and methods: We report here on the first introduction in cerebellum research of ion mobility separation (IMS) mass spectrometry for a systematic mapping of cerebellar gangliosides and determination of the species associated to development and aging. For this purpose, gangliosides extracted from post-mortem tissue biopsies of fetal -in the 15 and 40 gestational weeks (samples 15GW and 40GW)- and 65 years of age (sample 65Y) cerebellum were analyzed by nanoESI IMS MS in the negative ion mode under identical conditions.

Results: Altogether, no less than 694 molecular ions corresponding to 522 gangliosides, fucogangliosides and species modified by *O*-acetylation, *O*-GalNAc and CH_3COO^- were identified by IMS MS separation and screening, which represents almost five times more cerebellar structures than ever reported before.

Conclusions: The comparative analysis revealed for the first time that: i) 40GW contains the highest number of species (356), followed by 15GW (303) and 65Y (201); ii) fetal cerebellum gangliosidome is characterized by a much higher sialylation degree and species altered by carbohydrate and non-carbohydrate type of modifications than the gangliosidome of aged cerebellum; iii) significant developmentally- and age-regulated changes in the expression and structure of cerebellum gangliosides exist. These variations are to be correlated in the future with the neurological diseases, leading to the discovery of pathways to more effective therapeutic schemes.

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The pharmacological profile of natural compounds from *Aconitum* sp

Mernea Maria [a,*], **Mares Catalina** [a], **Avram Speranta** [a]

a) Department of Anatomy, Animal Physiology and Biophysics, Faculty of Biology, University of Bucharest, Splaiul Independentei 91-95, 050095, Bucharest, Romania;
**email: maria.mernea@bio.unibuc.ro*

Introduction: Plants from *Aconitum* genus are well known for their toxic effects. At the same time, their active compounds were shown to have beneficial effects like anticancer, antimicrobial, antifungal, or antioxidant [1]. A thorough characterization of these compounds is needed in order to understand their pharmacokinetic, pharmacodynamic and toxicity profiles. Here we used bioinformatics methods to address these aspects in the case of 21 constituents of *Aconitum* sp that were identified by high performance liquid chromatography (HPLC) [1].

Materials and methods: The SMILES structures of compounds were retrieved from PubChem database. MOE program was used to calculate the physico-chemical features of compounds relevant for their pharmacologic profile. The SMILES structures were loaded in Expasy server to evaluate the drug-likeness profiles of compounds. AdmetSAR was used to predict their ADMET properties and SwissTarget was used to predict their targets.

Results: From the 21 compounds, 15 present drug-like features. Except for magnoforine, the compounds should present a good gastro-intestinal absorption. The oral bioavailability is increased for ferulic acid, caffeic acid, delphinidin and syringic acid. The blood brain barrier permeability is increased for aconitine, hypaconitine, mesaconitine, magnophorine, gallic acid, ferulic acid, malvidin and syringic acid.

In what concerns the toxicity of compounds, most compounds are not toxic for birds or bees, have no mutagenic potential, but are toxic for fish. The prediction show that most compounds are hepatotoxic, except for hypaconitine, catechin, ferulic acid, caffeic acid, chlorogenic acid, epicatechin, coumaric acid or syringic acid. Except for gallic acid, ferulic acid, caffeic acid, coumaric acid, syringic acid, rutin and naringenin, the compounds could present toxicity at the mitochondrial level. Most compounds are expected to present toxicity at the respiratory level, except for gallic acid, ferulic acid, caffeic acid, coumaric acid. The compounds were not predicted to be cardiotoxic.

The predicted targets of *Aconitum* sp compounds pointed toward carbonic anhydrases of different types. In addition, some compounds like coumaric acid, daidzein and genistein modulate estrogen receptors. Other compounds like genistein, hypaconitine and magnoflorine should modulate targets from the central nervous system.

Conclusions: The compounds from *Aconitum* sp appear as promising therapeutic agents, acting on carbonic anhydrases and other targets even from the central nervous system. Some compounds present favorable pharmacokinetic and toxicity profiles, but there are compounds presenting different types of toxicity like hepatotoxicity, respiratory or mitochondrial.

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Glicolipidomics of human glioblastoma multiforme by ion mobility mass spectrometry

Mirela Sarbu[a,*], **David E. Clemmer**[b], **Željka Vukelić**[c], **Alina D. Zamfir**[a,d]

a) Department of Condensed Matter, National Institute for Research and Development in Electrochemistry and Condensed Matter, Timisoara, Romania;
b) Department of Chemistry, The College of Arts & Science, Indiana University, Bloomington, Indiana, USA;
c) Department of Chemistry and Biochemistry, Faculty of Medicine, University of Zagreb, 10000, Croatia;
d) Department of Technical and Natural Sciences, "Aurel Vlaicu" University of Arad, Arad, Romania;
**email: mirela.sarbu86@yahoo.co.uk*

Introduction: Glioblastoma multiforme (GBM) is the most widespread primary brain tumor in adults. With a rapid infiltration rate into the nearby tissue, GBM has drawn a significant attention because of its poor prognosis and the limited treatment options available. The research nowadays is focused on the determination of the molecular mechanisms related to GBM tumor invasion and the discovery of innovative approaches for invasiveness suppression. Since gangliosides (GGs) are tumor-associated antigens, we have introduced here IMS MS for the discovery of GBM-specific structures [1].

Materials and methods: GGs were extracted from a brain tumor localized in the frontotemporal cortex of the right hemisphere in a male patient, age 47. The histopathological analysis has shown that the tumor is GBM, grade IV. Ten µL of the working GBM GG solution in methanol were infused into a Synapt G2S instrument. To enhance the GG separation, the IMS parameters were set as follows: ESI voltage 1.5kV, cone voltage 45 V, IMS wave velocity 650 m/s, IMS wave height 40 V and collision energy 40 eV.

Results: The 2D data set of GBM GGs revealed the GG separation into mobility families based on their charge state, carbohydrate chain length, and the degree of sialylation. IMS MS offered a reliable separation, given the detection and identification in GBM of 215 ions, corresponding to no less than 160 distinct glycoforms [1], more than triple the number of GGs previously discriminated in GBM with no separation prior to MS [2]. The tumor was found dominated in equal and high proportions by GD3 and GT1 forms, with a particular incidence of C24:1 fatty acids in the ceramide. By the occurrence of only one mobility feature and the diagnostic fragment ions, the IMS tandem MS conducted using collision-induced dissociation (CID) disclosed for the first time the presence of GT1c(d18:1/24:1) newly proposed here as a potential GBM marker.

Conclusions: By IMS MS and CID MS/MS various novel species could be identified and added to the currently existing panel of glioblastoma tissue-associated structures, since the number of the GGs identified here is three times higher than ever discovered in this tumor type.

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New strategies for potentiating the effect of tissue transglutaminase-fibronectin small molecule inhibitors in ovarian cancer treatment

Monica Tudor [a,*], **Cristian Munteanu** [a#], **Gabriela Chirițoiu** [a#], **Florin Jipa** [b], **Ștefana M. Petrescu** [a], **Gary E.Schiltz** [c,d,e], **Daniela Matei** [e,f,g], **Livia E. Sima** [a]

a) Institute of Biochemistry of the Romanian Academy, 296 Splaiul Independentei, Bucharest, Romania;
b) National Institute of Laser Plasma and Radiation Physics, 409 Atomistilor Street, Bucharest-Magurele, Romania;
c) Northwestern University, 303 E Superior Street, Chicago, USA; d) Jesse Brown VA Medical Center, 820 S Damen Ave, Chicago, USA
**email: monica.tudor@biochim.ro # These authors contributed equally*

Introduction: Ovarian cancer (OC) is the 8th most common cancer in women. It is often diagnosed at late stages - 60% of cases are present with stage III or IV cancer [1] and this affects the response to treatment. Tissue transglutaminase (TG2), an overexpressed protein in OC tumors [2], interacts with fibronectin (FN) and promotes OC intraperitoneal dissemination, which makes TG2 a promising target for treatment. We previously identified MT4 as a promising small molecule inhibitor (SMI) of TG2-FN protein-protein interaction [3]. We recently developed 5 new MT4 analogues (#2997, #2998, #3002, #3010, #3011) and investigated their effect on OC cells. We used phosphoproteomics to analyze the adaptive signaling of OC cells treated with MT4 in order to identify new potential TG2 co-targets.

Materials and methods: We used MTS assay to evaluate SMIs cytotoxicity. Cells were seeded in 3D culture conditions to evaluate spheroid formation. For early attachment assessment, we determined DNA content of cells attached to FN within 45 min. Wound healing was performed to evaluate cell migration. SILAC labeled cells were used in a 30 minutes adhesion on FN. Both attached and suspension cells were collected, lysed and prepared for LC-MS/MS analysis using Easy nanoLC II coupled with LTQ- Orbitrap Velos Pro Mass Spectrometer. Data analysis was performed using Proteome Discoverer V 1.4, KEGG pathways and IPA. Synergy was evaluated using CompuSyn analysis. A gradient generating microfluidic chip was obtained using laser-based methods for testing the effect of various inhibitor concentrations or combinations thereof on OC cells or spheroids attachment onto FN. Live cell imaging of mesothelial clearance was performed using primary LP9 mesothelial cell monolayers and SKOV3 spheroids.

Results: To characterize the newly developed SMIs, we tested their effect on spheroid formation using OVCAR5 cell line and its *Tgm2* gene CRISPR knockout variant. Compound #3011 totally prevented sphere formation, similar to *Tgm2* gene excision. Early attachment assay revealed that SMIs #3002 and #3011 diminished cell adhesion. Wound healing assay showed that #2997 inhibited SKOV3 cell migration. We developed a microfluidic chip that allowed us to perform real-time visualization of mesothelial clearance. The results revealed that our proposed SMIs had an inhibitory effect on SKOV3 spheroids' capacity to displace mesothelial cells. Phosphoproteomic analysis revealed two classes of upregulated proteins – sirtuins and p21 activated kinases (PAKs). SIRT1 inhibitor molecules in combination with MT4 or #3011 affected 3D spheroid formation, number of 2D colonies formed, and had a cytotoxic effect on OVCAR5 cells. MT4-PAK1 inhibitors combinations enhanced OVCAR5 cell adhesion inhibitory effect of MT4.

Conclusions: We obtained new SMIs for TG2-FN interaction with improved capacity to inhibit spheroid formation and OC cell attachment. We then identified new promising molecular TG2 co-targets for OC treatment that induce cell death in both 2D and 3D conditions.

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Absolute and Relative Expression Analysis of Plasma MicroRNAs in Patients with Prostate Cancer – A Comparison

Paula Diana Ciordas [a,b,*], **Mirabela Romanescu** [a,b], **Aimee Rodica Chis** [b],
Catalin Marian [b], **Ioan Ovidiu Sirbu** [b]

a) Doctoral School, "Victor Babes" University of Medicine and Pharmacy, ''Eftimie Murgu'' Square
300041 Timișoara, Romania*;*
b) Discipline of Biochemistry, "Victor Babes" University of Medicine and Pharmacy, ''Eftimie Murgu''
Square 300041 Timișoara, Romania
**email:* paula.muntean@umft.ro

Introduction: Prostate cancer (PCa) is the second most common malignancy in men and a major source of male mortality. Due to their outstanding stability in various biological fluids, microRNAs are excellent diagnostic and prognostic biomarker candidates in various cancers (including PCa). Plasma microRNAs quantification involves real-time PCR, through either a relative or an absolute approach. Despite a rigorous methodology for choosing the right normalizer, relative quantification might lead to results drastically different from those obtained by absolute quantification. Herein we compared the findings from absolute (using standard curve) and relative quantification experiments conducted on both the guide and the passenger strand of 5 microRNAs known for their involvement in PCa [1-3].

Materials and methods: Total RNA was purified from plasma specimens collected from PCa patients and controls. *Caenorhabditis elegans* miR-39 mimic was spiked-in during the extraction procedure for subsequent external normalisation. Next, we used TaqMan assays to conduct singleplex qRT-PCR reactions for each microRNA of interest. For absolute quantification, standard curves were generated using 10 log dilutions of synthetic RNA oligonucleotides. Carrier RNA from bacteriophage MS2 was used to mimic the RNA plasma profile and improve the recovery of short and low-represented RNA species. Data analysis and visualizations were performed on RStudio integrated development environment.

Results: Our findings show that compared to absolute quantification, the results from relative quantification experiments are less consistent and more variable, most probably due to variability in the expression of reference genes. Furthermore, we present data indicating that the GC content of target microRNA could influence de quantification outcome and should be taken into consideration when designing art-PCR quantification experiments. Thus, while more labour-intensive and time-consuming, standard-curve absolute quantification stands out as the more reliable method of measuring the plasma level of microRNAs.

Conclusions: Dysregulation of the circulating microRNAs profile of PCa patients could be exploited as potential diagnosis tool for the disease. Absolute quantification using a standard curve is a suitable technique for quantifying small RNA-based plasma biomarkers since it provides highly reproducible results and allows for the determination of the exact copy number of species of interest.

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Profiling and structural characterization of gangliosides in human temporal lobe epilepsy

<u>Raluca Ica</u> [a], Kristina Mlinac-Jerković [b], Svjetlana Kalanj-Bognar [b], Cristian V.A. Munteanu [c], Andrei J. Petrescu [c], Alina D. Zamfir [a,d]

a) Department of Condensed Matter, National Institute for Research and Development in Electrochemistry and Condensed Matter, Timisoara, Romania;
b) Department for Chemistry and Biochemistry & Croatian Institute for Brain Research, School of Medicine, Zagreb University, Croatia;
c) Institute of Biochemistry of the Romanian Academy, Bucuresti, Romania;
d) Department of Technical and Natural Sciences, "Aurel Vlaicu" University of Arad, Romania;
**email: raluca.ica@gmail.com*

Introduction: Temporal lobe epilepsy (TLE) is one of the most common forms of epilepsy in humans. The molecular phenomena underlying seizures involve variations in membrane ion homeostasis leading to disturbances in neuronal excitability [1]. Numerous pathophysiological effects of gangliosides have been demonstrated, but their potential role in the molecular pathogenesis of TLE has not been explored. We developed a high-resolution (HR) MS^n approach using Orbitrap MS nanoESI to assess changes in the ganglioside pattern in TLE-affected adult hippocampus compared to normal hippocampus, with the aim of detecting TLE-associated structures.

Materials and methods: The native ganglioside mixtures analyzed were purified from human hippocampus affected by TLE (sample HipE) and age-matched normal hippocampus tissue (sample Hip), used as the control. Gangliosides were extracted in chloroform: methanol: water (1:2:0.75 by vol.), separated, collected and purified. The MS experiments were conducted in the negative ion mode on a LTQ Orbitrap Velos equipped with nanoESI source. Multistage mass spectrometry (MS^n) was carried out by higher energy collision dissociation (HCD) at variable collision energies within 35-80 eV.

Results: The high sensitivity and the resolution employed in the present experiments enabled the detection and identification of no less than 99 different gangliosides in hippocampus affected by TLE and 75 in the normal adult hippocampus specimen, which represent 5 times more structures than ever reported in the adult tissue and the highest number of species identified in hippocampus solely on the basis of MS profiling. The results also showed marked differences of the ganglioside expression in TLE vs. control, particularly with respect to the sialylation degree of components, discovered as a general marker of TLE. Another major finding was the occurrence of tetrasialo-fucogangliosides in TLE and species modified by either *O*-acetylation or CH_3COO^-. Additional structural analysis by nanoESI high-energy collisional dissociation (HCD) MS/MS gave rise to fragmentation patterns supporting the presence of GQ1b (d18:1/18:0) isomer as a novel TLE marker.

Conclusions: In this study high resolution MS approach provided detailed data on altered hippocampal gangliosidome in TLE, establishing GQ1b as a specific TLE-related biomarker, and strongly supported the hypothesized association of gangliosides with disturbed ion homeostasis underlying seizures pathogenesis in humans.

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Evaluation of total iodine uptake in biofortified chilli peppers

Ana Varadi [a,*], Claudia Cimpoiu [a] and Augustin C. Mot[a]

[a]*Faculty of Chemistry, Department of Chemistry, Babes-Bolyai University, Cluj-Napoca, Romania*
**email:* ana.varadi@ubbcluj.ro

Introduction: Among human micronutrient deficiencies, iodine is unique for the fact that its deficiency appears regardless of the economic status of the population, being prevalent in developing as well as developed countries. Iodized salt approach has several drawbacks [1], that is why other methods have to be used in order to supply the individuals with adequate amounts, one of them being iodine biofortification of vegetables. Considering that chilli peppers cannot be consumed in high quantities because of their pungency as well as either deficit or sudden iodine excess can be dangerous for human health [2], we decided to evaluate the amount of iodine that can be internalized in chili pepper fruits after iodine biofortification and without toxic effects for the plant.

Materials and Methods: Chili peppers varieties or cultivars of the *Capsicum annuum* and *Capsicum baccatum*, were biofortified with iodine as KI beginning with the 25th day from germination and were divided in two experimental approaches. For the first experimental design iodine was added in three doses namely 2.5, 6.5 and 16 mg iodine per kg soil, doses that were divided in 9 weeks. For the second design, iodine doses were 25, 50 and 100 mg I/kg soil and were divided in 4 weeks. When ripen, the fruits, leaves and roots were oven-dried at 45 ˚C for 72 h, milled into powder and iodine was analysed using the ferric thiocyanate-nitric acid catalytic kinetic method, following the sample combustion.

Results: The recovered iodine in soil was about 50% of the added amount, the other 50% being volatilised and internalised in the plants. Iodine content internalised in plants decreased in the order roots > leaves > fruits. High difference of iodine uptake into fruits was noted among the tested varieties (between 1 and 4 mg I/kg dried fruit for the first experimental design and between 3 and 11 mg I/kg dried fruit for the second experimental design).

Conclusions: Only a half of the iodine biofortification dose remains in soil. The iodine biofortification degree in chilli peppers is highly dependent upon the used varieties.

Keywords: biofortification, chili peppers, iodine

In vitro and *in vivo* assessment of the antioxidant properties of *Apis melifera* venom

Camelia Alina Gîlcescu (Florescu), George Bica, Elena Camelia Stănciulescu, Loredana Colhon, Cătălina Gabriela Pisoschi*

University of Medicine and Pharmacy of Craiova, 2-4 Petru Rares, 200349, Craiova, Dolj, Romania
**email: c_pisoschi@yahoo.com*

Introduction: The virtues of some bee products (pollen, honey) have been recognized for centuries. Among bee products, the venom has been intensively studied in the last decades to obtain definite scientific data on potential therapeutic properties associated with its complex composition. Research is focused especially on its anti-inflammatory effect, but antioxidant and antimicrobial activities are still under debate. The experiments were designed to assess *in vitro* antioxidant activity of two samples of bee venom (BV) from *Apis melifera* and its *in vivo* effect on the antioxidant status in a rat model of FCA-induced arthritis.

Materials and methods: 2, 2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid, ABTS) and 1, 1'-diphenyl-2-picrylhydrazyl (DPPH) assays were used to determine *in vitro* antioxidant activity of BV samples. *In vivo* antioxidant effects of bee venom were evaluated after induction of arthritis on male Wistar rats following an intraarticular injection of 120 µL of Freund's complete adjuvant (FCA). Rats were randomised in four groups: two control groups (negative and positive-arthritic), one treated with methotrexate as a standard drug for rheumatoid arthritis and the fourth group including animals receiving BV (2 mg/kg). Blood reduced glutathione level, glutathione peroxidase and catalase activities as well as serum total antioxidant capacity and lipid peroxides were measured using spectrophotometric standardized methods.

Results: The differences between the antioxidant activity of the two BV samples were statistically significant in both DPPH and ABTS assays. IC_{50} values for both free radical scavenging methods were lower for sample BV2, this proving to have a better antioxidant effect than sample BV1. Both BV samples proved a free radical scavenging effect more than 50%. Arthritic rats had increased levels of lipid peroxides and decreased blood glutathione, glutathione peroxidase and catalase activities compared to sham and those receiving methotrexate. Bee venom treatment significantly increased the levels of reduced glutathione, glutathione peroxidase and catalase activity and decreased lipid peroxidation.

Conclusions: Bee venom has been shown to improve the antioxidant defence in rats with FCA-induced arthritis due to own antioxidant properties. No doubt, the antioxidant involvement sustains its well-studied anti-inflammatory effect.

Antioxidant and antiproliferative activity of anthocyanin rich extract from roses (*Rosa* L.)

Cosmin-Alin Faur [a,*], **Marius Zăhan** [a], **Gabriela Roman** [b], **Claudiu Ioan Bunea** [c], **Eugenia Hârșan** [b], **Andrea Bunea** [a]

a) Faculty of Animal Science and Biotechnology, University of Agricultural Sciences and Veterinary Medicine, Calea Mănăștur 3-5, Cluj-Napoca, Romania;
b) The Horticultural Research Station, str. Horticultorilor 5, Cluj-Napoca, Romania;
c) Faculty of Horticulture and Rural Development Business, Calea Mănăștur 3-5, Cluj-Napoca, Romania;
**email: alincosminfaur@yahoo.com*

Introduction: Rose petals (*Rosa* L.) are a rich source of bioactive compounds, including anthocyanins. Anthocyanins and anthocyanidins are water-soluble compounds well-known for their antiproliferative and antioxidant properties, which are closely related to their structure and redox properties [1]. However, only a limited number of studies have investigated the antiproliferative activity of rose extracts on cell cultures [2,3]. The main objective of this study was to perform a biochemical analysis of several rose varieties and select the varieties with the highest anthocyanin content in the petals for testing their antiproliferative activity on two epithelial cell lines.

Materials and methods: In this study, 18 rose varieties were examined to identify anthocyanins, determine total polyphenols, total flavonoids, total lipids and antioxidant activity using specific methods for each class of compounds. The three rose varieties with the highest anthocyanin content were chosen to evaluate their antiproliferative activity on two human skin tissue-derived cell lines. The cell lines used were a tumor cell line (A375), which produces malignant melanoma, and a normal cell line (Hs27), to assess the effect of five different concentrations of anthocyanin-rich extract (200-1000 µg/mL).

Results: The results showed the presence of 9 anthocyanins through HPLC analysis, with the highest concentration of anthocyanins found in the varieties Porta Nigra, Lili Marleen and Schwarze Madonna. These varieties maintained their position regarding total polyphenol and total flavonoid concentrations, as well as antioxidant activity. Moreover, the MTT test conducted to determine antiproliferative activity showed only antiproliferative effects on the A375 tumor cell line, regardless of the varieties and concentrations used. On the other hand, on the normal cell line Hs27, concentrations up to 1000 µg/mL for Porta Nigra and Lili Marleen varieties and up to 600 µg/mL for the Schwarze Madonna variety did not affect the integrity of normal cells.

Conclusions: The anthocyanins from the three rose extracts demonstrated the ability to inhibit cell proliferation in the A375 tumor cell line without affecting cells from the normal Hs27 cell line, in a concentration-dependent manner.

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Valorization of carotenoid from sea buckthorn pomace using green solvents and ultrasound-assisted extraction

Cristina Gherasim [a*], **Adela Pintea** [a]

a) University of Agricultural Sciences and Veterinary Medicine, Department of Chemistry and Biochemistry, 3-5
Calea Mănăştur Street, Cluj-Napoca, Romania
**email: cristina.gherasim@usamvcluj.ro*

Introduction: Sea buckthorn pomace is a by-product of fruit juice production wich still contains large amounts of carotenoids and other lipophilic compounds. In order to limit the negative impact that organic solvents have on the environment and on the human health, there is a growing interest for developing new extraction techniques for lipophilic compounds. Beside the use of green solvents, effficient methods like ultrasound-assisted, microwave-assisted or enzyme-assisted extractions are envisaged. Encapsulation of biologically active compounds has gained interest because this method can improve both the stability and the bioaccessibility of natural compounds, providing viable solutions for food and pharmaceutical industry.

Materials and methods: Sea buckthorn fruits were cold pressed, resulting the juice and the pomace fractions. The resulting pomace was then weigthed, dried and grinded to produce a powder. Carotenoid extraction was conducted comparatively, using an organic solvent mixture (ethyl-acetate:methanol: petroleum ether) and green solvents (ethyl lactate ethyl acetate, methyl tetrahydrofuran, cyclopentyl methyl ether, sunflower oil). Two extraction methods were applied: maceration (solid-liquid extraction) and ultrasound-assisted extraction. The carotenoid extract was used for encapsulation using alginate and calcium chloride. The capsules (wet or dehydrated) were added to yogurt samples and then subjected to *in vitro* digestion using the INFOGEST method in order to determine the bioaccessibility of carotenoids. Carotenoid content in all samples was determined by spectrophotometry and C30-HPLC-PDA, using external calibration with commercial standards.

Results: The main carotenoids identified in pomace extracts were β-carotene, γ-carotene, lycopene, zeaxanthin, zeaxanthin myristate-palmitate, and zeaxanthin dipalmitate. The highest extraction yield (99.34±8.9%) was obtained using ultrasound-assisted extraction with MeTHF as extraction solvent. The final extract was dissolved in sunflower oil and used to obtain the alginate microcapsules. The capsules had a mean diameter of 600 µm and the encapsiulation efficiency was 99.4%. The retention of carotenoids in microcapsules after 30 days of storage, at 4°C, was 77 % for wet beads and 74% for dehydrated beads, which indicates a good stability. The bioaccessibility of carotenoids from yogurt samples supplemented with alginate microcapsules was 29.5±3.9% (wet) respectively 40.7±3.8 (dehydrated), compared to control 42.1±4.6% (wet) respectively 40.8±4% (dehydrated). Better results were obtained when yogurt was supplemented with dehydrated capsules compared to wet capsules.

Conclusions: The results presented in this study shows that green solvents and ultrasound-assisted extractions are efficient methods for valorization of sea buckthorn pomace. Encapsulation of carotenoid extract in alginate microcapsules have a protective effect and does not impair their bioaccessibility in a food model.

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Toxicity of Gadolinium on *Stevia rebaudiana* grown *in vitro*

Florina Violeta Scurtu [a,c], Doina Clapa [a], Floricuța Ranga [b], Vasile Coman [b], Augustin C. Moț [c], Sonia Ancuţa Socaci [b], Loredana Florina Leopold [b], Cristina Coman [b]*

a) Life Sciences Institute, University of Agricultural Sciences and Veterinary Medicine, 3-5 Calea Mănăstur, 400372, Cluj-Napoca, Romania;
b) Faculty of Food Science and Technology, University of Agricultural Sciences and Veterinary Medicine, 3-5 Calea Mănăștur, 400372, Cluj-Napoca, Romania; Faculty of Chemistry and Chemical Engineering, Babes-Bolyai University, Arany János 11, 400028 Cluj-Napoca, Romania;
**email: cristina.coman@usamvcluj.ro*

Introduction: Lately, there is increasing interest in the potential toxicity of different widely used nanomaterials or chemicals on the environment and on edible plants. Gadolinium (Gd)-based contrast agents (e.g., gadobutrol - GB) are still extensively used for diagnostic purposes in magnetic resonance imaging. However, there are several issues related to their use. For example, after administration, they are excreted and end up in hospital effluents and, consequently, in wastewaters [1]. As wastewater treatment is not flawless, Gd presence in tap water and tap water-based beverages (e.g., Coca-Cola from fast-food franchises in big cities in Germany) has been documented recently [2,3].

In this study, we have assessed the accumulation of Gd ions on *Stevia rebaudiana* plantlets grown *in vitro*, and their impact on plant growth and on plant metabolites (chlorophylls, carotenoids, ascorbic and dehydroascorbic acids). The plants were exposed to GB in concentrations up to 3 mM.

Materials and methods: The plant metabolites were quantified using high performance liquid chromatography (HPLC). Oxidative stress was assessed by the malondialdehyde (MDA) assay and electron paramagnetic resonance (EPR).

Results: At the highest doses (1 and 3 mM), GB exposure had detrimental effects, leading to significant inhibition in plant development. The concentrations of carotenoids, chlorophylls A and B (Chl A and B), were severely decreased at these high exposures. For the highest dose, Chl A and B concentrations descended by 52.76% and 58.92%, and the levels of lutein, zeaxanthin, and beta-carotene were inhibited by 38.16%, 54.34%, and 54.34%, respectively. A detrimental effect of the high GB doses was also seen via the increase in free radical species detected by EPR, which was negatively correlated with the evolution of antioxidants (i.e., ascorbic acids). The 0.1 mM dose of GB can be considered a threshold, below which the plant defense system is functional, and the GB toxicity seemed to be low or moderate.

Conclusions: Given all these findings, the photosynthesis at high concentrations of GB was impacted significantly. Such pronounced changes in plant morphology and levels of antioxidant molecules can be related to the plant's stress response, which could be sometimes unnoticeable at low exposures and harmful above certain thresholds.

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Allium sativum and *Allium ursinum* extracts: antimicrobial activity and phytochemical characterization

Ioana Andreea Barbu [a,b,c*], Alexandra Ciorîță [a,d], Rahela Carpa [a,c,e], Augustin Catalin Moț [f], Anca Butiuc-Keul [a,b,c] and Marcel Pârvu [a,c]

a) Faculty of Biology and Geology, Babeș-Bolyai University, 1, M. Koganiceanu Street, 400084 Cluj-Napoca, Romania; ioana.barbu@ubbcluj.ro (I.A.B.); alexandra.ciorita@ubbcluj.ro (A.C.); anca.keul@ubbcluj.ro (A.B.-K.); marcel.parvu@ubbcluj.ro (M.P.)
b) Doctoral School of Integrative Biology, Babeș-Bolyai University, 400015 Cluj-Napoca, Romania
c) Center for Systems Biology, Biodiversity and Bioresources, Babeş-Bolyai University, 400084, Cluj-Napoca, Cluj, Romania
d) National Institute for Research and Development of Isotopic and Molecular Technologies, 400293 Cluj-Napoca, Cluj, Romania
e) Institute for Research-Development-Innovation in Applied Natural Sciences, Babes-Bolyai University, 30 Fântânele St., Cluj-Napoca, 400294 Romania
f) Faculty of Chemistry and Chemical Engineering, Babes-Bolyai University, 11 Arany Janos Street, 400028 Cluj-Napoca, Romania; augustin.mot@ubbcluj.ro
**email: ioana.barbu@ubbcluj.ro*

Introduction: *Allium* plants have been known for their antimicrobial properties since ancient times. Recent studies have shown that the leaves and bulbs of these plants are rich in phenols, flavonoids and thiosulfinates, compounds that have proven antimicrobial, antioxidant or antitumor activity. Naturally, in *Allium* bulbs or leaves, allicin is formed after crushing or cutting the plant organs under the action of the allinase enzyme. The chemical composition of the extracts depends on the species. [1, 2].

Materials and methods: The antimicrobial activity of the extracts was determined by the agar disk diffusion method. The minimum inhibitory concentration (MIC) and minimum bactericid concentration (MBC) were also determined. Through the phytochemical analysis of the extracts, the determination of three classes of compounds was followed: the total thiosulfinates content, the total phenolic content and the total flavonoid content.

Results: The antimicrobial activity of *A. sativum* and *A. ursinum* extracts against *Escherichia coli, Staphylococcus aureus, Candida albicans* and *Candida parapsilosis* can be correlated with the presence of large amounts of thiosulfinates. Both extracts have shown better results against *Candida* species (inhibition zones of 20–35 mm) and against Gram-positive bacteria, *Staphylococcus aureus* (inhibition zones of 15–25 mm) compared to the other two species tested. The MICs for the tested microbial species were similar to the MBCs. There were also discrepancies between MICs and MBCs, e.g., for the, *A. ursinum* extract on *C. parapsilosis* (MIC = 6.25 and MBC = 25) and for the *A. sativum* extract on *E. coli* (MIC = 6.25 and MBC = 25). The two extracts have a similar content of thiosulfinates compounds. The *A. sativum* extract has double the amount of flavonoids compared to that of *A. ursinum*, while the total phenolic content is higher in *A. ursinum*.

Conclusions: These results demonstrate the antimicrobial effect of the extracts suggesting their use as adjuvant treatment for microbial infections.

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Assessing the risks of nanoplastic exposure to plants: Implications for growth and steviol glycoside production

Loredana F. Leopold[* a,b], **Florina V. Scurtu** [b], **Doina Clapa** [b], **Nicolae Leopold** [c], **Stefania D. Iancu** [c], **Floricuța Ranga** [a], **Sonia A. Socaci** [a,b], **Cristina Coman** [a,b]

a) Faculty of Food Science and Technology, University of Agricultural Sciences and Veterinary Medicine, 400372 Cluj-Napoca, Romania;
b) Life Sciences Institute, University of Agricultural Sciences and Veterinary Medicine, 400372 Cluj-Napoca, Romania;
c) Faculty of Physics, Babeș-Bolyai University, 400084 Cluj-Napoca, Romania
**email: loredana.leopold@usamvcluj.ro*

Introduction: This study aims to investigate the toxicity of polystyrene nanoplastic (NP) on *Stevia rebaudiana* plants and develop a quick detection method for NP accumulation in plant tissues using dark field microscopy with hyperspectral imaging (HSI). The research focuses on the potential risks associated with NPs exposure to plants.

Materials and methods: For this, *in vitro* and *in vivo Stevia rebaudiana* plants were exposed to NPs at concentrations up to 250 mg/L to evaluate their impact on plant growth, morphology, and metabolites. High-Performance Liquid Chromatography (HPLC) was used to quantify the *Stevia rebaudiana* metabolites.

Results: HPLC data indicates that the concentration of various plant metabolites decreased with the NP dose (up to 250 mg/L NP), which can be attributed to the stress response of the plant caused by NPs. The growth of *Stevia rebaudiana* plantlets was negatively impacted by polystyrene NP. However, the production of steviol glycosides, the natural sweeteners in stevia, exhibited a biphasic dose-response, suggesting hormesis, with the highest values at 250 mg/L NP (a 1.3-fold increase compared to controls).

Conclusions: These findings highlight the potential risks associated with NP exposure to plants and suggest the need for further research on the effects of NPs on plant growth and productivity.

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Antioxidant and cytoprotective effects of *Sorbus aucuparia L.* fruit extract in gentamicin-induced nephrotoxicity on mice renal cells

Mara AURORI [a,*], **Daniela HANGANU** [b], **Emoke PALL** [a], **Mihai CENARIU** [a] **and Sanda ANDREI** [a]

a) Faculty of Veterinary Medicine, University of Agricultural Sciences and Veterinary Medicine, 400374 Cluj-Napoca, Romania;
b) Department of Pharmacology, Faculty of Pharmacy, University of Medicine and Pharmacy "Iuliu Haţieganu",400372 Cluj-Napoca, Romania
**email: mara.aurori@usamvcluj.ro*

Introduction: *Sorbus aucuparia L.*, also referred to as mountain ash or European rowanberry, has lately been identified as a significant source of several bioactive components, being historically used in the treatment of various ailments. As such, these berries contain great concentrations of organic acids (particularly ascorbic acid), polyphenols (especially phenolic acids - chlorogenic and neochlorogenic acids), carotenoids and microelements [1,2]. Gentamicin is an antibacterial drug that is frequently utilized in medical facilities due to its extensive range of activity against gram-negative bacteria. It additionally causes unfavorable adverse reactions, such as ototoxicity and nephrotoxicity [3]. Therefore, the aim of this study was to investigate the *in vitro* antioxidant and nephroprotective effects of rowanberries, with the latter potentially leading to the discovery of novel therapeutics for kidney disease.

Materials and methods: Firstly, two types of ethanolic extracts were obtained. They were characterized by employing chromatographic methods. The antioxidant capacity was evaluated using DHPP and FRAP assays. As for cytotoxic activity, primary renal cell cultures were obtained from mouse fetuses' renal tissue, which was further treated using a mixed method including tissue explants and enzymatic treatment. The obtained cell cultures were treated with gentamicin (100 µg/µL), *Sorbus aucuparia* extract in three distinct concentrations (4.8 µg/µL, 10.7 µg/µL and 19.1 µg/µL) and with a combination of both. Furthermore, MTT colorimetric assay and Annexin V-FITC cell apoptosis detection kit were employed to determine the extract's potential cytoprotective effect.

Results: The analysis of the *Sorbus aucuparia* ethanolic extracts revealed that the total phenolic content was 1.003 ± 0.046 mg GAE/mL and the total carotenoid content was 95.68 ± 0.297 µg/g. The antioxidant capacity of *Sorbus aucuparia* showed a significant activity, with DHPP = 24.51 mg/mL and FRAP = 0.016 µmol Trolox/mL extract. The MTT and Annexin V-FITC tests revealed that the extract-treated groups had higher cell viability than the antibiotic-treated group at the first two concentrations. Additionally, increased viability was observed in the groups treated with extract and antibiotic, which was comparable to the extract-treated cells. In contrast, a significant decline in cell viability was observed at the highest concentration (19.1 µg/µL) when compared to untreated cells. Moreover, this concentration registered a significant lower viability in extract-treated and extract+antibiotic-treated groups in comparison to cells that received gentamicin alone. These results could be attributed to the extract and gentamicin's additive pro-oxidant effects.

Conclusions: The *Sorbus aucuparia* fruit extract demonstrated a significant *in vitro* antioxidant capacity and, with the exception of the highest dose, it also demonstrated cytoprotective effects in gentamicin-induced stress on mice renal epithelial cells.

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The effect of *Rhizophagus irregularis* on phenolics and essential oil of the *Echinacea purpurea* (L.) Moench

Martin IAKAB [a,b*], **Erzsébet DOMOKOS** [b], **Csilla ALBERT** [c], **Béla BIRÓ-JANKA** [b], **Francisc V. DULF** [a]

a) University of Agricultural Sciences and Veterinary Medicine, Faculty of Agriculture, 3-5 Mănăștur Str, 400372, Cluj-Napoca;
b) Sapientia Hungarian University of Transylvania, Faculty of Technical and Human Sciences Târgu Mureș, Calea Sighișoarei nr. 2, 540485, Târgu Mureș;
c) Sapientia Hungarian University of Transylvania, Faculty of Economics, Socio-Human Sciences and Engineering, Piaţa Libertăţii nr. 1., 530104, Miercurea Ciuc
**email: jakab.marton@ms.sapientia.ro*

Introduction: The majority of studies conducted on *Echinacea purpurea* regarding elicitor (e.g. arbuscular-mycorrhizal fungi – AMF) induction have been carried out *in vitro*, using controlled conditions. The application of elicitors had conducted to elevated levels of various secondary metabolites [1-3]. The aim of this study was to test the competitive ability of *Rhizophagus irregularis* on different substrates (greenhouse and open field conditions) in the presence of local microorganism communities, in order to induce quantitative and qualitative changes in the active principles of *E. purpurea*.

Materials and methods: Greenhouse and an open field experiment were conducted with *E. purpurea* inoculated with *R. Irregularis* and control plants. In the greenhouse exepriment three different soil types and sterile peat were used. HPLC analyses for phenolic compounds were made on dried leaves of *E. purpurea* plants. The dried *herba* was analysed with GC-MS for volatile oil composition.

Results: According to the HPLC analyses six phenolic acids were detected. The two dominant compounds were chicoric and chlorogenic acids. Control plants presented significantly higher values in phenolic content compared to treated plants, on sterile peat and open field stagnic Luviosol in case of caftaric, chlorogenic, caffeic, chicoric acids, and echinacoside. AMF colonization rate favored caftaric, chlorogenic and chicoric acids content on several substrates. The quantitative analyses of the essential oil showed significantly higher yield for potted control plants on stagnic Luviosol. The qualitative analyses revealed 35 constituents, the most abundant were caryophyllene oxide and germacrene D. According to the PCA analyses the essential oil obtained from treated plants contained γ-cadinene and humulene epoxide 2 in higher proportions, while the essential oil of the control plants was abundant in spatulenol and shyobunol.

Conclusions: Observations on AMF effect on root essential oil yield and composition would be of high interest. Further studies with other AMF species in combination with growth-promoting bacteria should be carried out, to assess their competitive behavior with the native microorganisms.

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Antioxidant supplementation and its impact on inflammatory and oxidative stress parameters in diet-induced obesity rats

Mihaela Cotul[a, *], **Daria-Antonia Dumitraș** [a], **Alexandra Dreancă** [a], **Andrei Paul Ungur** [a], **Sanda Andrei** [a]

a) Faculty of Veterinary Medicine, University of Agricultural Sciences and Veterinary Medicine,
Calea Mănăștur 3-5, Cluj-Napoca, Romania;
**email: mihaela.cotul@usamvcluj.ro*

Introduction: Obesity represents a significant public health challenge, associated with the development of various chronic conditions, such as diabetes, cardiovascular disorders, and cancer [1]. In response to this escalating issue, dietary supplements, particularly antioxidants, have gained attention for their potential role in obesity prevention and treatment. Nonetheless, the precise efficacy of these supplements in mitigating or attenuating obesity remains uncertain [2,3].

The primary objective of this investigation was to induce obesity in six distinct groups of rats and assess the impact of antioxidant supplementation within two distinct dietary regimens: a normal diet (ND) and a high-fat diet (HFD). Specifically, the diets were supplemented with either plain flaxseed oil or flaxseed oil enriched with powdered antioxidants sourced from *Calendula officinalis* and *Ribes nigrum*.

Materials and methods: In this experimental study, 32 male Sprague-Dawley rats were selected and subjected to an 8-week duration experiment. The experimental animals were divided into six groups and subjected to specific dietary interventions. Three groups received a normal diet (ND), while another three groups were fed a high-fat diet (HFD). Within each diet group, subgroups were further established, with some animals receiving antioxidant powder in flaxseed oil at doses of 200 mg/kg *Calendula officinalis* (CO) and 200 mg/kg *Ribes nigrum* (RN). The administration of the antioxidant powder in flaxseed oil was performed daily via gavage the entire experimental period.

At the end of the study, whole blood and tissue samples were obtained from the rats for the assessment of various markers associated with inflammation (interleukin IL-6 and TNF-α) and oxidative stress (total antioxidant capacity-TAC, catalase activity-CAT, lipids peroxides-LPO, superoxide dismutase-SOD and 8-hydroxy-2'-deoxyguanosine-8-OH-dG). Subsequently, a comparative analysis was performed to evaluate the differences in these markers between the groups receiving antioxidant supplementation and the non-supplemented control groups.

Results: The consumption of a high-fat diet (HFD) resulted in a substantial elevation in body weight, as indicated by an increased body mass index (BMI) ranging from 0.84 to 0.92. Additionally, the HFD led to a notable accumulation of adipose tissue, reflected by an adiposity index ranging from 2.8% to 3.5%. These changes were accompanied by significantly heightened levels of oxidative stress and inflammation. However, the administration of antioxidants demonstrated a significant mitigating effect on the HFD-induced body weight gain, reducing it by 14% along with a decrease in BMI (0.3 g/cm^2). Furthermore, the antioxidant powder exhibited favorable effects on the inflammatory status, manifesting a significant reduction in inflammation. Moreover, it exerted a notable antioxidant effect by modulating the activity of catalase (CAT) and reducing the levels of lipid peroxidation (LPO) and 8-hydroxy-2'-deoxyguanosine (8-OH-dG).

Conclusions: The findings of this investigation have offered valuable elucidation regarding the prospective role of antioxidant supplementation in the prevention or mitigation of obesity.

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The impact of silica nanoparticles (sinps) on some biochemical parameters in *Silybum marianum* seedlings

Popovici Lucia-Florina[1], **Oprică Lăcrămioara-Anca**[2*]

a) Angelmed Laboratory, 700127 Iasi, Romania;
b) Faculty of Biology, Alexandru Ioan Cuza University of Iasi, 700506 Iasi, Romania;
**email: lucia.florina97@yahoo.ro*

Introduction: Currently, agricultural productivity is declining due to the negative effects of climate change, rising global temperatures and increased environmental stress. Increased soil salinity is known to be one of the most devastating processes leading to a drop in agricultural productivity, causing major reductions in cultivated area and limiting the productivity and quality of crop plants, due to increased osmotic stress, nutritional disorders and toxicity[1]. Therefore, finding methods to combat salt stress in plants is very important. One of the most promising methods capable of neutralizing the harmful effects of soil salinity is the creation, selection and use of nanoparticles (NPs) with strong antioxidant effects. In this sense, numerous studies suggest that Silicon Nanoparticles (SiNPs) can penetrate into plant cells and influence their metabolic activities [2].

Materials and methods: For this study, we used 60 seeds of *Silybum marianum* (Secuieni Agricultural Research and Development Station, Neamţ, Romania) which were divided into 6 experimental groups of ten seeds each (Control; Control + 50 mM NaCl; Control + 100 mM NaCl; SiNPs; SiNPs + 50 mM NaCl; SiNPs + 100 mM NaCl). Immediately after applying the first treatment, their growth was monitored for 22 days. After that, the seedlings were homogenized and processed to determinethe activity of some biochemical parameters like Superoxide dismutase (SOD), Catalase (CAT), Peroxidase (POD), the Malonaldehyde level (MDA), as well as the content of assimilatory pigments in the leaves [3].

Results: The results of our study indicate that salt stress in concentrations of 50 mM and 100 mM, together with SiNPs, intensifies the antioxidant defense system in *S. marianum* seedlings, compared to variants treated only with NaCl.In contrast, the treatment with SiNPs managed to successfully reduce the oxidative damage of NaCl treatment on *S. marianum*, by restoring the activity of antioxidant enzymes (SOD, CAT, POD) and by down-regulating the level of MDA. Aslo, the total amount of assimilatory pigments (chlorophyll a, chlorophyll b and carotenes) from the leaves, at the thistle, showed a notable decrease, under the action of the applied treatments, which seem to inhibit their biosynthesis.

Conclusions: The changes in the physiological and biochemical parameters obtained by determinations at the foliar level, of the *S. marianum* species, constitute an important starting point for the standardization of some toxicity tests in the evaluation of the SiNPs treatment of plants. Further study is needed to elucidate how SiNPs initiate these effects.

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The GC-MS analysis of some volatile oils and the evaluation of their antibacterial properties against *S.aureus* and *E. coli* bacterial strains

Roberta Tripon [a,c*], **Cristina Gașpar** [b], **Dorin Camen** [a], **Mihaela Enea** [a,c], **Ramona Românu** [a,c], **Camelia Tulcan** [a,c]

[a)] *Faculty of Engineering and Applied Technologies, University of Life Sciences "King Michael I"from Timișoara;*

[b)] *Faculty of Veterinary Medicine, University of Life Sciences "King Michael I" from Timișoara;*

[c]*ULST Research Institute for Biosafety and Bioengineering (ICBB) Timișoara*
**email: roberta.tripon@usvt.ro*

Introduction: During the last decade, the use of natural products for their pharmacological properties has gained great attention worldwide. This increased interest in some phytoproducts is correlated especially with their ability to limit antibiotic resistance, being a great alternative to some conventional antibacterial products. In this context, this study aimed to analyze by GC-MS the chemical composition of some volatile oils and to evaluate their antibacterial potential against two bacterial strains of great interest, *Escherichia coli* DK0336 and *Staphylococcus aureus* CP8150.

Materials and methods: The tested volatile oils were represented by thyme volatile oils (*Thymus sepryllum* and *Thymus vulgaris*) and grapefruit volatile oils (*Citrus × paradisi*), all of which were purchased from authorized bio-producers from Romania. For the identification of the biologically active volatile compounds in the composition of the oils, GC-MS chromatographic analysis was carried out, using a 7890A gas chromatograph (Agilent Technologies, USA) coupled with a triple quadrupole mass detector, 5975C (Agilent Technologies, USA) and equipped with a HP-5MS capillary column (30m × 0.25mm × 0.25m). The determination of the inhibition percent (IP%) of these oils on the *E. coli* DK0336 strain, respectively *S. aureus* CP8150 strain, was carried out by the broth microdilution method in microtitre plates. This method is one of the most widely used techniques for assessing the susceptibility of bacteria to certain products with bacteriostatic and/or bactericidal effects.

Results: *T. vulgaris* and *T. serpyllum* volatile oils demonstrated a remarkable effect on the two bacterial strains (*S. aureus* CP8150 and *E. coli* DK0336), indicating inhibition percentages (IP%) of up to 99.36%. These high values of the IP (%) were manifested especially at small volumes of oil (0.5 and 1.0 µL). Regarding the *Citrus × paradisi* oils, however, the inhibition percentages did not exceed the value of 45.42%. At higher volumes (1.5 and 2.0 µL) a potentiation of bacterial development was observed on the *S. aureus* CP8150 bacterial strain.

Conclusions: The tested volatile oils showed important values of the inhibition percent (PI%), especially at small volumes. Due to the presence of terpenic structures such as thymol and carvacrol in their composition, *T. vulgaris* oils demonstrated the highest PI (%) values, respectively 99.36% for the *E. coli* strain and 99.31% for the *S. aureus* strain.

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Polyphenols and antimicrobian activity in glycerol *Sanguisorba officinalis* L. and *Sanguisorba minor* Scop. Roots extracts

Alexandra-Cristina Tocai (Moţoc) [a,*], **Simona Ioana Vicas** [b]

a) Doctoral School of Biomedical Science, University of Oradea, 410087 Oradea, Romania;
b) Faculty of Environmental Protection, University of Oradea, 410048 Oradea, Romania;
**email: tocai.alexandra@gmail.com*

Introduction: *Sanguisorba officinalis* L. and *Sanguisorba minor* Scop. are wild edibles species belonging to the Rosaceae family used frequently in traditional medicine but even now they are being researched more and more. From our previous study [1], where we characterized and compared the ethanolic extracts of *Sanguisorba* species to highlight which species is more valuable according to the phenolic profile and antimicrobial activity we wanted to find an alternative method, more environmentally friendly for the polyphenol's extraction. The main aim of this research was to investigate if glycerol is an efficient solvent for extraction polyphenols from the roots of *S. officinalis* L. and *S. minor* Scop. The antibacterial activity of glycerol extracts of medicinal plants was also investigated.

Materials and methods: The selected medicinal plants were collected from Bihor and Cluj country, Romania. *S. officinalis* L. roots were collected in August 2021 from Săcădat village, Bihor county and *S. minor* Scop. Roots were collected in May 2021 from Bucea village, Cluj county. A specimen of each *Sanguisorba* ssp. was kept in the Herbarium of the faculty of Medicine and Pharmacy Oradea, Romania, registered in NYBG Steere Herbarium, under the code: Uop 05 367-*S. minor* Scop. and Uop 05 368- *S. officinalis* L. An alcohol-free extract based on *Sanguisorba officinalis* roots purchased commercially was also investigated. One gram each of *S. officinalis* and *S. minor* was macerated in a water-glycerol combination (60%) at room temperature for 20 days, with daily shaking for 10 minutes, twice a day, to extract the phenolic compounds. All experiments were conducted in triplicate. The phenolic profile of glycol extracts from the roots of the two medicinal plants, as well as the commercial product, was determined using HPLC-DAD-ESI-MS (ESI$^+$). The spectral values were recorded in the 200-600 nm range for all peaks.The chromatograms were recorded at the wavelength λ = 280, 340 and 520 nm. For MS, full scan ESI positive ionization mode was used in the following working conditions: capillary voltage 3000 V, temperature 350 °C, nitrogen flow 7 l/min and *m/z* 120-1200. A variation of the Kirby-Bauer diffusion was used for evaluation of antimicrobial activity against *Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa.*

Results:. According to the HPLC analysis, ellagic acid and catechin are the major components of the *Sanguisorba* extracts, with higher amounts detected in the root extract of *S. minor* Scop. In terms of antimicrobial activity, the glycerol extract of *S. minor* Scop. roots shown high antibacterial activity against all tested bacteria, particularly *E. coli*, with an inhibition zone of 12.03 ± 0.35 mm. Glycerin extract from *S. minor* Scop. had the highest polyphenol concentration and antibacterial activity, followed by *S. officinalis* L. glycerin extract, and the commercial product investigated.

Conclusions: The potential of water/glycerol combinations to efficiently extract polyphenols from the roots of *Sanguisorba* species was demonstrated in this study, highlighting the fact that the roots of *S. minor* Scop. are rich in phenolic compounds, and the glyceric extract has an effective antibacterial action against *E. coli*.

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In vitro **antioxidant and antigenotoxic potential of** ***Viscum album*** **L. subsp.** ***Album***

Vicas Simona Ioana [a,*], **Laslo Vasile**[a], **Klezken Eva** [b], **Memete Adriana Ramona** [a],
Fertig Tudor Emanuel [c,e], **Marta Daciana Silvia** [c,d]

a) Faculty of Environmental Protection, University of Oradea, 26 Gen. Magheru Street, 410048 Oradea, Romania;
b) University of Oradea, Doctoral School of Biomedical Science, 1 Universitatii Street, 410087 Oradea, Romania;
c) Ultrastructural Pathology and Bioimaging Lab, Victor Babeș National Institute of Pathology, 99-101 Splaiul Independentei Street, 050096 Bucharest, Romania;
**email: svicas@uoradea.ro*

Introduction: Nowadays, the mistletoe (*V. album* L.) continues to attract a lot of interest, particularly for research into the bioactivity of its components. A variety of chemical substances, ranging from small molecules like phenolic acids and flavonoids to proteins with high molecular weights like lectins and viscotoxins, are found in the *V. album* . The greater part of mistletoe research is focused on cancer treatment [1]. Most oncological patients apply *V. album* L. extracts to alleviate symptoms related with the disease and therapy, as well as to improve quality of life. Mistletoe extracts have been shown to have anticancer, pro-apoptotic, anti-proliferative, and immune-modulatory properties[1]. Recent researches have also indicated that the antioxidant capacity of mistletoe extracts is affected by the host tree [2]. The major aim was to emphasize the reduction of genotoxicity caused by hydrogen peroxide in normal human dermal fibroblasts (NHDF). Furthermore, the ability of mistletoe aqueous extract to synthesize selenium nanoparticles (SeNPs) has been highlighted.

Materials and methods: Mistletoe leaves have been collected from apple (*Malus domestica*) (host tree) in May 2022 and characterised in terms of polyphenols content using HPLC-MS-ESI. Antioxidant capacity of mistletoe leaves was evidence by four different methods such as DPPH, FRAP, TEAC and CUPRAC. The characterization of SeNPs was performed by UV-Vis spectrum, and DLS, while morphological details were observed by TEM analysis (Negative-Stain). NHDF and the specific culture kit (Fibro-blast Growth Medium-2 BulletKit) were used for the cell culture treatments. Neutral single cell gel electrophoresis, known as Comet assay, was performed according to Purcarea et al.2022[3].

Results: Twenty-one compounds have been separated and tentatively assigned based on their retention durations, UV absorption spectrum, m/z values, and major fragments. In mistletoe leaves, hydroxycinnamic acids dominate hydroxybenzoic acids. Chlorogenic acid was the major phenolic acid in mistletoe leaves (1.413±0.11). Twelve molecules were found from the flavonoids class, from which quercetin derivatives were predominant. Among the four antioxidant methods used, it was evident that mistletoe extract has antioxidant properties. In addition, the synthesis of SeNPs was confirmed by the appearance of red color confirming the reducing ability of mistletoe. The SeNPs showed a maximum absorption at 270 nm and the apparent zeta potential was recorded at a maximum value of -24.5 mV, which indicates that these nanoparticles do not form aggregates in solution leading to a stable dispersion. According to TEM, the spherical shape was confirmed and the diameter of SeNPs was around 130 nm. To highlight the potential of mistletoe extract to reduce genotoxicity induced by hydrogen peroxide, two types of treatments were implemented: one of them was pre-treatment to highlight the possible role of DNA protection before the application of hydrogen peroxide (75 µM) and another, post-treatment to highlight DNA repair potential. Our results showed a significant reduction in the frequency of DNA damage, quantified by %DNA tail, in cells treated with mistletoe extract (25 and 50 µg/ml) in both treatment, suggesting a bioantimutagenic behavior of the extracts

Conclusions: Mistletoe is a medicinal plant that has significant health benefits. The ability of the extract to reduce the selenite salt to elemental selenium has been demonstrated in this work for the first time in the literature. The mistletoe's protecting but also repairing impact on DNA from fibroblasts degraded by

H2O2 exposure opens up novel opportunities to explore the molecular mechanisms underlying these features.

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Strategy for deorphanizing G-protein coupled receptor 75 using brain extract fractions

Cosmin Trif[a,*], **Sorin Tunaru**[b]

a) Institute of Biochemistry of the Romanian Academy, Romania, Cell Signaling Group
**email: trifcosmin@gmail.com*

Introduction: G-protein coupled receptors (GPCRs) are a large superfamily of transmembrane proteins that convert extracellular stimuli into cellular effects. Given their vast implication in physiological and pathological processes they are targeted by almost a third of FDA approved drugs. G-protein coupled receptor 75 (GPR75) is member of this family with high expression in the retina, brain and pancreas and with implications in metabolic syndrome, diabetes and obesity. Although some studies suggest as endogenous ligands for GPR75, 20-Hydroxyeicosatetraenoic acid (20-HETE) and the chemokine RANTES, the receptor is still considered orphan. Given its expression pattern in human and mouse, we devised it is a good strategy for its deorphanization by using brain extracts.

Materials and methods: The brain extracts were obtained by homogenizing mouse brains in 10 volumes of a solution containing 70% V/V acetone 1 M acetic acid, and 20 mM HCl. The homogenate was then delipidated with dyethyl ether. The aqeous phase was loaded into a reverse phase column Waters Sep-Pak C18 20 cc Vac Cartridge and we eluted the fractions using an acetonitrile gradient ranging from 0% to 100%. The fractions and the lipid phase was liophylized and diluted into HBSS and their biological activity was tested in HEK 293T cells transfected with empty vector or GPR75 and a genetic probe for measuring intracellular calcium and cAMP.

Results: Using our pharmacological aproach we could not detect 20-HETE and RANTES as ligands for GPR75. The brain extract fractions contained a good yield of protein ranging from 0.5 mg/ml up to more than 5 mg/ml. Some of this fractions were biological active in calcium and cAMP assays but not selective for GPR75

Conclusions: In this study we optimized a method for obtaining biological active fractions from mouse brain that can be used to discover novel endogenous ligands for orphan GPCRs like GPR75.

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Interactions between transient receptor potential subfamily ankyrin member 1 (TRPA1) and the epidermal growth factor receptor (EGFR) in glioblastoma

David-Ionuț Băcioiu, Florentina Piciu, Alina-Ana-Maria Botezatu, Dana Cucu

Department of Anatomy, Animal Physiology and Biophysics, Faculty of Biology, University of Bucharest,
Splaiul Independentei 91-95, 050095 Bucharest, Romania
**email: bacioiu.david-ionut22@s.bio.unibuc.ro*

Introduction: Cancer is one of the leading causes of worldwide mortality, alongside diabetes and cardiovascular diseases. Although not as common as other malignant neoplasms (2-3 cases per 100,000 people), glioblastoma makes up for the relative lack of incidence in its severity. This disease is characterized by a low survival rate upon diagnosis, a favorable switch of microglial genetic mechanisms, as well as upregulation and mutations within key proteins (PDGFR, EGFR, EGFRvIII, PTEN, p53). EGFRvIII, for example, renders a resistant tumoral phenotype, taking into account that EGFRvIII cells promote the growth of neighboring wtEGFR cells in an IL-6, LIF and gp130-dependent mechanism. Transient Receptor Potential (TRP) channels emerge as tumor markers and putative therapeutic targets from this barren landscape. The mammalian TRP channel superfamily encompasses six families with 28 members, translating into 27 proteins and one pseudogene (TRPC2). Among them, TRPA1 stands out as one of the most versatile receptors. The channel's activity can be modulated by a wide range of compounds and stimuli, including Ca^{2+} ions, pro-inflammatory mediators, ROS and RNS, xenobiotics, and temperature. Regarding its *modus operandi* in tumor cells, the channel does not follow a prototypical model, inducing somewhat different effects depending on the cancer subtype. It appears to be involved in lung cancer development [1], whereas TRPA1 activation in PDAC cell lines reduces motility [2]. Factors like the polymodal character and the ubiquitous expression turn the family of TRP channels into a fertile research topic.

Materials and methods: To fully understand the interaction between EGFRvIII and TRPA1 in the glioblastoma landscape, our research group will resort to a battery of assays. We shall address the functionality of TRPA1 in the U-87MG_WT and U-87MG_EGFRvIII cell lines electrophysiologically, with a series of whole cell patch clamp recordings in the presence of specific agonists and antagonists (AITC, ROS, and A-967079). Furthermore, the wound healing assay will be used to evaluate cell motility of tumor cells treated with adequate EC50 AITC, ROS, and A-967079 concentrations.

Results: Considering the duality of activated TRPA1 in tumoral subtypes, its role in glioblastoma cell lines must be experimentally addressed first. However, certain clues in the scientific literature point to an anti-tumoral function of TRPA1 [3]. It should be observed that both WT and EGFRvIII$^{+/+}$ cells express slower migration patterns in the wound healing assay, with some speculative differences attributable to EGFRvIII. TRPA1 might also become upregulated after ROS stimulation or by still undescribed EGFR/EGFRvIII - TRPA1 interactions, statement which allows for further investigations.

Conclusions: Our theories, if proven true, hint at the involvement of TRPA1 in glioblastoma cell apoptosis. Given that TRPA1 activation reduces glioblastoma cell motility, pharmacological targeting of TRPA1 could become a noteworthy option.

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Molecular aggregation pathway of cataract-associated γD-crystallin p23T congenital defect

E Matei [a,*], A Filip [b], C G Floare [a], A Pirnau [a], M Mic [a]

a) National Institute for Research and Development of Isotopic and Molecular Technologies,
67-103 Donat, 400293 Cluj-Napoca, Romania
b) Enzymology and Applied Biocatalysis Research Centre, Faculty of Chemistry and Chemical Engineering,
Babeş-Bolyai University of Cluj-Napoca, Arany János Str. 11, RO-400028 Cluj-Napoca, Romania
**email: elena.matei@itim-cj.ro*

Introduction: Cataract, the most common cause of blindness, which represents a disturbance of the delicate balance required for transparency, either because of age-related degenerative modifications, or genetic mutations, causing abnormal aggregation of eye lens crystalline proteins. In order to understand how structural changes in the P23T variant of human γD-Crystallin (hγD-Crys) lead to congenital cataract, we carry out comparative protein-protein interaction studies between wild-type hγD-Crys, and hγD-P23T, with small heat shock chaperone αB-Crys, by solution NMR. We plan to get new insights into the structure of hγD-P23T aggregates, and its interaction mechanism inside the eye lens, with potential interference in protein aggregation by small molecules. These valuable findings will provide alternatives to reduce the severity, delay, or treat congenital cataract [1-3].

Materials and methods: The synthetic DNA encoding the wild-type hγD-Crys, the hγD-p23T mutant, and αB-Crys were individually inserted into the pET-14b expression vector, and transformed into BL21(DE3) cells. Proteins were expressed either in LB culture medium, or modified M9 medium for isotopically ^{15}N-labeled hγD-Crys, the hγD-p23T mutant, and αB-Crys. Proteins were purified by ion exchange, and size-exclusion chromatography using Q-FF, SP-FF, and Superdex columns, attached to the FPLC-Äkta system. For protein-protein interaction study we used ^{1}H-^{15}N HSQC NMR pulse sequence.

Results: For studying hγD/hγD-p23T:αB-Crys complex, we monitor the chemical shift perturbations in the ^{1}H-^{15}N HSQC spectra of ^{15}N-labeled hγD-Crys/hγD-p23T in complex with unlabeled αB-Crys. The superposition of ^{15}N- hγD-p23T HSQC spectra in the absence and presence of unlabeled αB-Crys, provides the first quality control of the complex, in addition to exchange regime, and binding interface. The spectral changes on HSQC spectra of ^{15}N-hγD-Crys/hγD-p23T upon addition of unlabeled αB-Crys, does not indicate a visible perturbation of the chemical shifts, only a reduction in the intensity of the NH signals is observed, as a result of the broadening effect of the resonance lines. This fact indicates a non-specific interaction between hγD-Crys, respectively hγD-p23T, with the chaperone protein αB-Crys.

Conclusions: Therefore, the soluble (monomeric) fraction of the hγD-p23T mutant interacts non-specifically with the chaperone protein αB-Crys, in a manner similar to the wild-type hγD-Crys, suggesting only a transient interaction. This is the first NMR study to highlight the protective role of the chaperone protein αB-Crys, ensuring proper three-dimensional folding of partner proteins (crystallins) present in the (human) eye lens. However, this non-specific interaction is not sufficient to prevent the aggregation process of the hγD-p23T mutant, under physiological conditions. Apparently, the macromolecular self-association process of the hγD-p23T mutant is a pronounced one, so that it manages to escape from the supervision of the chaperone αB-Crys, causing cataract.

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Modelling of MHC-I Peptide Loading Complex

Floriana Sibel Bectaş[a,*], **Teodor Asvadur Şulea**[a], **Laurentiu Spiridon**[a], **Andrei J. Petrescu**[a]

a) Department of Bioinformatics and Structural Biochemistry, Institute of Biochemistry of the Romanian Academy, Splaiul Independentei 296, Bucharest 060031, Romania
**email: floriana.bectas@biochim.ro*

Introduction: The major histocompatibility complex class-I (MHC-I) peptide-loading complex (PLC) is a critical multi-protein complex of the immune system composed of no less than 5 proteins forming one module that dimerize into the endoplasmic reticulum. Each of the two modules of the PLC complex consists of five components: Tapasin, Calreticulin, ERp57 and the two proteins, namely Human Leukocyte Antigen (HLA) and β2-microglobulin that together form the Major Histocompatibility Complex Class-I (MHC-I). In this dimeric form the PLC controls the selection and loading of peptides onto MHC-I. After loading, the stable peptide-MHC-I complex is released and leaves the ER through the secretory pathway to reach the cell surface for antigen presentation. Here it is recognized by killer T cells which ensures the detection of infected or mutated cells by the immune system. The process flow that takes place in the PLC during loading is barely known due to the complexity and size of this system and its specific dynamics. However some breakthroughs have been recently made by Cryo-EM which resulted in several low-resolution structural descriptions of this molecular machine. Based on these templates, we present a model of the PLC starting from the HLA-B sequence (IMGT/HLA Acc No: HLA00132) in order to gain a more precise view of the HLA-B based PLC complex and investigate the dynamic events that take place during the loading of Tyrosinase antigenic peptide 280-EEYNSHQSL-288.

Materials and methods: The structure of one PLC module was built by homology modelling with Modeller. This module was then duplicated and the two objects were assembled using constrained docking based on the experimentally identified contacts as joining links between the two monomers in order to generate the overall PLC heterodimer. Two N-glycans were further added using Glycopack (Paduraru et al 2006): (1) the immature high-mannose G1M9 oligosaccharide added to N86 (MHC-I) and (2) a high-mannose glycan linked to N233 (Tapasin). The system was subjected to both implicit and explicit solvent molecule simulations. Implicit solvent was used for Hamiltonian Monte Carlo simulations coupled with Gibbs sampling implemented in the Robosample software package. For the explicit solvent simulations the transmembrane helices were anchored into a POPC membrane and the system was solvated and immersed in a water box with 15Å padding and a concentration of 0.15M Na+/Cl-.

Results: In order to gain insights into the molecular interactions within the PLC structural model, each protein was independently subjected to electrostatic potential calculations using APBS. Subsequently, the calculated potential was mapped onto their surfaces to detect potential enthalpic contributions to their binding affinity. Furthermore, an assessment of putative hydrophobic patches at their interfaces was conducted to evaluate possible additional contributions to interaction stability.

Following the molecular simulation, an analysis of Root Mean Square Deviation (RMSD) and Root Mean Square Fluctuation (RMSF) was conducted to evaluate the stability of the system and identify potential hotspots on the interaction surfaces. Based on the aforementioned analysis, a ranking was established to identify and prioritize the significant contacts observed at the protein contact surfaces.

Conclusions: Comprehending the dynamic nature of the PLC complex holds paramount importance in gaining insights into its functional behavior and potential conformational changes occurring during the peptide loading process. This understanding plays a vital role in elucidating the complex's structure, dynamics, and functionality, particularly within the context of peptide loading onto Major Histocompatibility Complex (MHC-I) molecules.

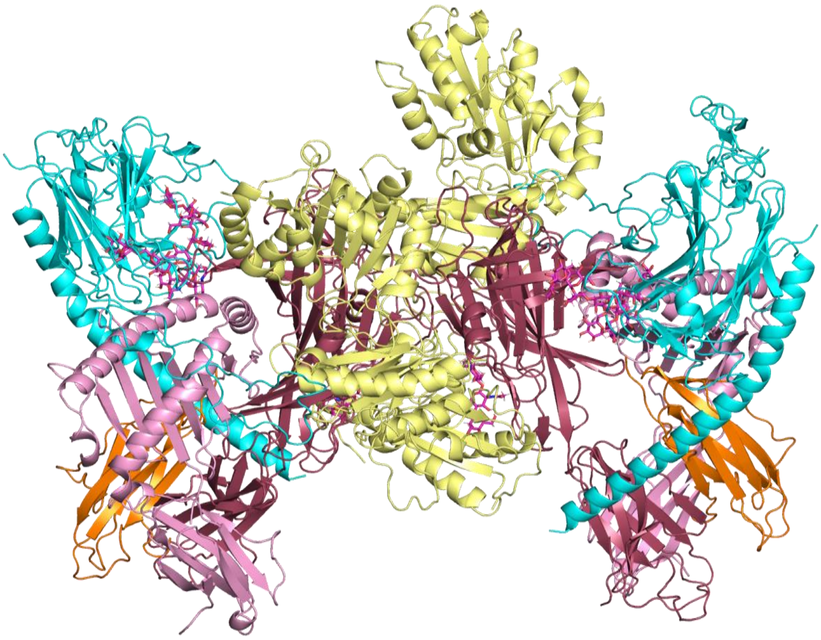

Fig. 1. PLC heterodimer model (Pink Cartoon - HLA (heavy chain of MHC-I); Orange Cartoon – β-2-microglobulin (light chain ofMHC-I); Cyan Cartoon - Calreticulin; Raspberry - Tapasin; ERp57 - Yellow Cartoon; Magenta Lines – Glycans. Image generated with Pymol v.2.2.3.

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High-throughput screening to identify inhibitors of RAGE-S100B interaction

Ioana Popa [a*], **Carmen Tanase** [a], **Bernhard Ellinger** [b], **Jeanette Reinshagen** [b], **Philip Gribbon** [b]

a) Institute of Biochemistry of the Romanian Academy, Splaiul Independentei 296, Bucharest, Romania
b) Fraunhofer Institute for Translational Medicine and Pharmacology ITMP ScreeningPort, Germany
**email: ipopa@biochim.ro*

Introduction: Receptor RAGE is an immunoglobulin-like transmembrane protein. Discrete sites on RAGE extracellular domains (V, C1 and C2 domains) bind various ligands, among which are advanced glycation end products, members of the S100 protein family, high mobility group box protein 1, amyloid β oligomers and fibrils, complement component C1q. Enhanced RAGE signalling pathways leading to pro-inflammatory and pro-oxidative cellular responses are induced by high concentrations of ligands and have been associated with pathological modifications in diabetes, peripheral neuropathy, cardiovascular diseases, chronic inflammation, cancer and neurodegeneration. Therefore, RAGE has been proposed as a promising disease modifying drug target for these pathologies (1). Growing evidences point to a role for RAGE and its ligand S100B in the pathophysiology of the brain, as active participants in the cellular processes which can have neurodegenerative effects (2). So far, screening campaigns to identify RAGE inhibitors focused on its interaction with amyloid β. Giving the prominent role of S100B as a cytokine in the nervous system, we initiated a high-throughput screen (HTS) to target S100B binding to RAGE.

Materials and methods: A time-resolved fluorescence resonance energy transfer (TR-FRET) assay was employed to measure RAGE-S100B interaction. The TR-FRET assay consisted of a His tagged VC1 fragment of RAGE coupled with anti-His antibodies labeled with Terbium as FRET donor and an FITC-labeled S100B protein as acceptor. Optimization of the TR-FRET reaction was done to select the most suitable assay set-up which was further used for the screening of a small commercial library of natural compounds (137 natural products), and for the HTS of an EU-OPENSCREEN chemical library (~100,000 compounds).

Results: The proteins used in the study (VC1-His, S100B, His-S100B) were expressed in bacteria and purified by affinity chromatography. Free amines of S100B and His-S100B were labeled with FITC. The buffer composition and the concentration of binding proteins and of the Terbium-labeled antibodies were optimized for both 96 well and 384 well microplate platforms. The affinity of S100B-FITC/ VC1-His binding was determined from the titration curves. The minimal concentrations that gave a signal-to-background ratio > 3 and a Z' factor > 0.7 were further used in competition experiments. Unlabeled-S100B, and an antagonistic RAGE peptide, but not BSA competed for the binding of S100B-FITC to VC1-His, proving that the assay set-up was specific for the interaction measurement. Our method was further tested in a small screen of a natural product and we identified three putative candidate inhibitors of RAGE/S100B interaction. The transfer of technology and further optimization of the protocol were done at the EU-OPENSCREEN site. The HTS was performed using 10μM of the tested compounds, and led to the initial identification of 875 hits. Of these, the active hits were validated in an additional confirmation run and in the counter-screen with His-S100B-FITC. Dose-response curves for all the identified compounds were profiled.

Conclusions: We developed a specific, robust and fast fluorescence-based method suitable for HTS which allowed the identification of small molecule candidates as new inhibitors of RAGE/S100B interaction. These compounds will be further tested in a relevant cell culture model for RAGE activation.

Acknowledgement: This work was funded from EU-OPENSCREEN Small Molecules Screening Call (PID 9521/2019)

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RP-HPLC studies on chiral separations of racemic nonapeptides sulfoxides

Lucian-Mihai Stănescu [a,*], **Corina Aramă** [a], **Ștefana Petrescu** [b], **Cristian V.A. Munteanu** [c]

a) University of Medicine and Pharmacy, Carol Davila, Faculty of Pharmacy, 6, Traian Vuia street, Bucharest, Romania
b) Department of Molecular Cell Biology, Institute of Biochemistry of the Romanian Academy (IBRA), 296, Splaiul Independenței, Bucharest, Romania
c) Department of Bioinformatics and Structural Biochemistry, Institute of Biochemistry of the Romanian Academy (IBRA), 296, Splaiul Independenței, Bucharest, Romania
**email: mihai.stanescu@umfcd.ro*

Introduction: The asymmetry is an intrinsic property of life. The biogenic molecules, with asymmetric carbon, the peptides and the proteins consist of mostly L-amino acids; only few contain D-amino acids in the structure. A particular example is related to the asymmetric sulphur atom, for peptides oxidised at methionine that form racemic sulfoxides with different biological properties comparing to unoxidised ones at methionine [1]. Previous studies reported chromatographic chiral separation for racemic R and S FMOC-L-methionine sulfoxide by supercritical fluid chromatography [2] and for crosslinked cysteine sulfoxide peptides [3], but not for underivatised peptides as is the case in this study. The aim of our study is to develop and validate a RP-HPLC enantioseparation method for the racemic mixture of the oxidised YM(Ox)DGTM(Ox)SQV, a linear nonapeptide containing two sulfoxide chiral centres, and to obtain enantiomeric pure fractions for further biological investigations.

Materials and methods: The analyte - the racemic peptide YM(Ox)DGTM(Ox)SQV oxidised at both methionines, (noted M(Ox)) was obtained by chemical synthesis. A JASCO HPLC equipped with a quaternary pump PU-2089 plus and a circularly dichroism detector CD-2095 plus was used for all the chromatographic experiments. The stationary phases used in the investigations of chiral separations were Lux i-Cellulose 5, Lux Cellulose 4 and Pirkle Covalent (R,R) Whelk O-1. The mobiles phases contained high percent of water and the organic solvents were acetonitrile, methanol, and isopropanol. The modifiers selected were CF3COOH and HCOOH as usually used for peptides separation in reversed-phase liquid chromatography. The experimental parameters were optimised to obtain the highest enantioselectivity.

Results: The chiral discrimination varies, depending on the stationary phase type, the organic solvent and the concentration of acid modifier. A partial separation of two of the 4 diastereomers was obtained with the stationary phase Lux Cellulose 4.

Conclusions: The obtained results can be optimised in order to collect the fraction of each enantiomers and then to test the potential use in therapeutics.

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The first complex of cobalamin with an organic peroxide

Maria Lehene [a,*], **Radu Silaghi-Dumitrescu** [a]

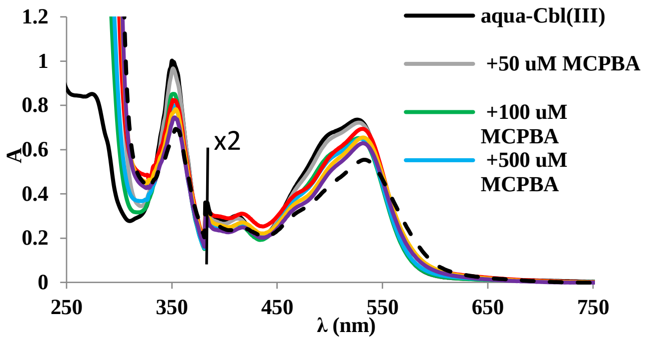
a) Department of Chemistry, Babeș-Bolyai University, Str. Arany Janos Nr. 11, RO-400028 Cluj-Napoca, Romania
**email: maria.lehene@ubbcluj.ro*

Introduction: Until recently, the reactivity of cobalamin with oxidizing agents has been confined to processes where, especially with strong oxidizing agents, the corrin ring is covalently modified by oxygenation or halogenation, or where Co(I) or Co(II) are oxidized to Co(III) in an outer-sphere manner [1, 2]. Aqua(III)cobalamin reacts m-chloroperoxybenzoic acid (MCPBA) to form a distinct and stable complex.

Materials and methods: UV-vis spectra were performed on a Cary 50 UV-vis spectrophotometer (Varian, Inc., Foster City, CA, USA). Stopped-flow spectra were collected on a Biologic SFM-300 system equipped with three syringes and sequential mixing, with a high-speed diode array detector. The NMR spectra were recorded at 22°C unless otherwise stated, after diluting the sample with D_2O (1:1 volume ratio), on a 400 MHz Bruker instrument. Raman spectra were measured at 22°C on a Renishaw inVia Raman spectrometer coupled with a Leica microscope. For DFT calculations, the Gaussian09 software package[17] was employed following the methodology previously tested and described for related Cbl complexes [3].

Results: As shown in Figure 1, upon reaction with MCPBA the UV-vis spectrum of aquacob(III)alamin (H_2OCbl^+) undergoes bathochromic shifts reminiscent of the hydroperoxocob(III)alamin adduct. Isosbestic points at 461 nm, 340 and 367 nm suggest formation of a single new product, which can in principle be assigned as a peroxoacid(MCPBA)-Cbl(III) complex analogous to the previously-reported cobalamin(III)-hydroperoxo. complex.

Conclusions: To conclude, Cbl(III) forms a relatively stable peroxoacid complex with deprotonated m-chloroperoxybenzoic acid, with distinct UV-vis and ^{1}H-NMR signatures. By contrast, tBuOOH does not appear to be able to form a peroxo complex with Cbl(III). These findings suggest that the peroxide coordination chemistry of cobalamin does extend beyond simply hydrogen peroxide,[5] but that not all peroxo compounds should be expected to bind to Cbl(III).



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Synthesis, degradation and trafficking processes of the GPR75 receptor

Ramona M. Tecucianu [a *], **Sorin Tunaru** [a] **and Ștefana M. Petrescu** [a]

a) Institute of Biochemistry of the Romanian Academy, Bucharest, Romania
**email:* tecram2605@yahoo.com

Introduction: G Protein-Coupled Receptor 75 is a receptor from the GPCR family, which represents the largest family of membrane proteins and the richest source of targets for the pharmaceutical industry. Recent studies have suggested that GPR75 has extremely high potential to be a drug target for diseases such as metabolic syndrome, obesity, dyslipidemia, diabetes, cardiovascular disease, and cerebrovascular disease [1]. Over time, several molecules such as RANTES (Regulated upon Activation, Normal T Cell Expressed and Presumably Secreted)/CCL5 (C-C Motif Chemokine Ligand 5) and 20-HETE (20-Hydroxyeicosatetraenoic acid) have been proposed as GPR75 receptor ligands. However, the Nomenclature and Standards Committee of the International Union of Basic and Clinical Pharmacology (NC-IUPHAR) continues to classify GPR75 as an orphan receptor. One of the most interesting finding related to GPR75 is that certain truncated variants of GPR75 (especially the truncated form at Gln234 of GPR75) were associated with a 1.8 kg/m2 lower body mass index (BMI), a higher body weight 5.3 kg less and 54% less chance of obesity in heterozygous carriers [2]. GPR75 might be involved in insulin secretion, potentiating secretion and preventing glucotoxicity-induced beta cell dysfunction [3]. So, we set out to study the processes of synthesis, trafficking and degradation of the GPR75 receptor and to investigate its role in insulin secretion.

Materials and methods: Firstly, the cellular localization of the GPR75 receptor was determined by an immunofluorescence experiment. In order to determine if the GPR75 receptor is glycosylated digestion with Endo H and PNGase F was performed. Then, the GPR75 receptor half-life was established by a cycloheximide chase assay. Furthermore, the degradation pathways of the GPR75 receptor were investigated by treatment with lysosomal/proteasomal inhibitors. Finally, the role of the GPR75 receptor in insulin secretion was explored through ELISA and Western Blotting techniques.

Results: The fluorescence microscope images revealed that the GPR75 receptor is predominantly present at the plasma membrane, but is also present in intracellular compartments. Treatment with Endo H and PNGase F of Hek293T cells transfected with GPR75 showed that most of the GPR75 receptor is glycosylated and transported to its final destination, while some remains retained in the ER. Treatment with cycloheximide led to the determination of the half-life of the GPR75 receptor, which is 4 hours. Moreover, it has been confirmed that the GPR75 receptor is degraded by the lysosomal pathway, but also by the proteasomal pathway. In addition, a decrease in the high-mannose form of GPR75 and an increase in the peptide form of GPR75 were observed with MG132 treatment. Regarding the role of GPR75 in insulin secretion, the results of the glucose stimulation experiments showed that in the case of silencing the GPR75 receptor there is an increase in the expression and secretion of proinsulin, respectively insulin.

Conclusions: This research investigated the processes of synthesis, trafficking and degradation of the GPR75 receptor and its role in insulin secretion. Future studies are needed to better understand the trafficking of the GPR75 receptor, such as identifying the cell organelles in which the receptor is present, as well as studying receptor internalization and recycling.

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Study on the interactions between icariside II and whey protein concentrate

Szabó Róbert[a, *], Rácz Csaba Pál[b], Dulf Francisc Vasile[a]

a) Department of Environmental and Plant Protection, University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Calea Mănăştur 3-5, 400372 Cluj-Napoca, Romania
b) Faculty of Chemistry and Chemical Engineering, Babeș-Bolyai University of Cluj-Napoca, Arany János 11, 400028 Cluj-Napoca, Romania
**email: robert.szabo@student.usamvcluj.ro*

Introduction: In the realm of health and wellness, the quest for natural compounds with significant health-promoting properties is a constant endeavor. Icariside II (ICS), derived from the *Herba Epimedii [1]*, has emerged as a compound of great importance for health promotion. With its diverse range of benefits and potential implications for various physiological processes, such as cardiovascular diseases, osteoporosis, or sexual dysfunction[2]. Icariside II is known to have relatively low bioavailability, meaning that it is not easily absorbed and utilized by the body when ingested. The cause is that ICS is not highly soluble in water[3], which hinders its absorption in the gastrointestinal tract. Low solubility limits its ability to dissolve and be transported into the bloodstream for systemic distribution. To overcome the challenges associated with the poor bioavailability of Icariside II, recent studies have explored various strategies. These include the use of drug delivery systems, such as nanoparticles or micelles, to enhance solubility and protect the compound from rapid metabolism. In our previous studies we have formed icariside II and whey protein concentrate (WPC) complexes, with the goal of achieving a higher water solubility, consequently significantly increase the bioavailability of ICS. This paper focuses on exploring binding mechanism of ICS-WPC complex.

Materials and methods: A pure batch of icariside II was sourced from Xi'an Day Natural Inc. (China). Our whey protein concentrate (80%) was purchased from Foodcom S.A. (Warsaw, Poland). All the other solvents used had analytical grade purity. All other reagents were of analytical grade.

The binding mechanism of the above-mentioned complexes was studied by X-ray powder diffraction, Fourier-transform infrared spectroscopy, and Differential scanning calorimetry and Molecular docking.

Results: In order to analyze the thermal profile of the ICS, WPC, and ICS-WPC physical mixtures and complexes, a Differential Scanning Calorimeter (DSC) was employed. From the DSC curves of the physical mixture the endothermic peak of the ICS can be spotted, while in the case of the complex this is not present. This indicates the success of the formation of ICS-WPC complexes. These findings were backed up with the XRD measurements, where similarly to the DSC, the intense peaks of ICS have disappeared in the complex. The difference between the physical mixture of ICS-WPC and the complex can be observed from the FTIR spectra. In this case between the ICS-WPC complex and the physical mixture a significant difference in the width of their peak in the 3600-3300 cm^{-1} can be observed. The peak in the case of the complexes are wider, meaning hydrogen bonds were formed. With the means of the molecular docking the interactions between the icariside II and β-lactoglobulin were investigated. As result the conformation with the lowest free energy was considered. This showed that three H-bonds were formed between the ICS and the protein, specifically the PRO-38 and LYS-69 amino acid residues. These are consistent with the results of FTIR measurements.

Conclusions: Measurements of XRD and DSC confirmed the formation of amorphous ICS-WPC complexes. The results of the FTIR and molecular docking showed evidence for the existence of H-bonds between the ICS and protein. Consequently, the formation of ICS-WPC complexes can be considered as a viable solution for increasing the bioavailability of the icariside II.

Acknowledgement: This work was supported by the Collegium Talentum Programme of Hungary

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