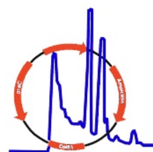

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Orphan G-protein coupled receptor 27 (GPR27), a novel and atypical adrenergic receptor

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Introduction: GPR27 is an orphan G-protein coupled receptor (GPCR) that belongs to the family of receptors known as "super-conserved receptors expressed in the brain (SREB)". The SREB family of receptors consists of three members, GPR27, GPR85, and GPR173. They share a high degree of sequence identity, are conserved throughout evolution, and are mainly expressed in the brain [1]. Despite extensive research in the last decades following their discovery no clear biological functions of the SREBs were assigned. GPR27 is one of the most interesting member of the SREB family as its potential role in tumorigenesis has been described. Moreover, GPR27 has been implicated in the regulation of glucose-stimulated insulin secretion, in a ligand independent manner. The aim of our study was to characterize the biological role of GPR27 by identifying its endogenous ligand(s).

Materials and methods: Human GPR27 receptor cDNA was cloned in pcDNA3.1 vector harboring an N-terminal FLAG-tag. For immunoblotting experiments and functional assays, GPR27 cDNA cloned in pcDNA3.1 vector was transiently transfected in HEK293T cells by using Lipofectamine 2000 reagent following manufacturer's indications. To determine intracellular calcium mobilization following GPR27 stimulation with compounds, HEK293T cells were cotransfected with GPR27 cDNA and a cytosolic localized calcium sensitive probe consisting of a fusion protein between aequorin and GFP, termend G5A. Similarly, to determine intracellular cAMP levels, a cytosolic probe sensitive to cAMP was expressed (p22F Glo, Promega). Chimeric receptors between GPR27 and the indicated receptors were generated through chemical synthesis (GenScript). Serum response element activity was measured using pGL4.33[luc2P/SRE/Hygro] (SRE-luciferase reporter gene assay), from Promega.

Results: Our initial results demonstrated that heterologous expression of GPR27 in various cell lines, including HEK293T cells, led to the specific and severe inhibition of several signaling pathways, such a adenylyl-cyclase-cAMP-CRE, or the inbition of Src-induced tyrosine phosphorylation of intracellular proteins. These effects were resembling a constitutively inhibitory activity, however in a G-protein independent manner. By replacing the C-terminal domain of GPR27 with the corresponding C-terminus of β1-adrenergic receptor, we obtained a chimeric receptor that displayed an identical pharmacological profile with β1-adrenergic receptor. This interesting finding led us to test whether GPR27 is able to mediate cellular effects of adrenergic ligands such as isoproterenol, adrenaline and noradrenaline. Although adrenergic ligands did not alter cAMP levels or CRE activity, they were able to specifically inhibit serum response element (SRE) in a GPR27- and concentration-dependent manner. Moreover, this effect was independent on G-proteins and arrestin signaling. Further experiments demonstrated that GPR27 mediates the inhibition of several receptor tyrosine kinases such as EGFR, as stimulation of cells expressing GPR27 with isoproterenol induced strong inhibition of EGF-induced SRE activation.

Conclusions: Our results demonstrated for the first time the existance of a novel adrenergic system, consisting of the orphan GPR27 receptor that lacks the ability to engage G-proteins and arrestins after adrenergic ligands stimulation. Moreover, GPR27/adrenergic ligand pairs are able to specifically inhibit the activity of a wide range of receptor tyrosine kinases. The discovery of such system opens novel research lines in cancer and developmental biology and has a high translational potential.

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

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Hepatitis C Virus antigen design and alternative production platforms for vaccine development

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Introduction: A highly efficient direct acting antiviral treatment for Hepatitis C Virus (HCV) infection set a revolutionary precedent in antiviral therapy. However, for the viral infection eradication a prophylactic vaccine would be required. That would help in achieving the WHO resolution of 65% mortality and 95% infection reduction by 2030. To accomplish that, new vaccine candidates and more affordable recombinant protein expression systems are needed. RNA enveloped viruses have common strategies for immune response evasion like genetic diversity, the glycan shield or structural flexibility of the envelope proteins regions targeted by neutralizing antibodies (nAb). Taking a reverse vaccinology approach, we designed and tested different interventions for the structural stabilization of a HCV E2 nAb epitope. Although state of the art in the industry, mammalian recombinant expression systems are very expensive. Particularly for Low Medium Income Countries more affordable vaccine production systems are needed. We have shown proof of concept for subunit vaccine production in plants and microalgae.

Materials and methods: Using an "in house" fast free energy sampling technology, structurarly stabilized HCV E2 antigens were suggested. The mutants were evaluated for their heterologous expression in HEK293 cells, ER exit capacity and differential binding to conformation dependent antibodies (beta hairpin Ap33; liniar epitope – 3/11). The prioritized mutant was expressed in Expi293F cells and the monomeric form was purified by IMAC followed by SEC. HCV E2 derived antigens were transiently expressed in lettuce, Nicotiana benthamiana or red algae *Porphyiridium* and were further purified by afffinity purirfication chromatography. Immunogenicity was evaluated in BALB/c and CD-1 mice either by intravenous (IV) or oral immunization. Sera neutralizing capacity was tested using an "in house" HTS pseudovirus system.

Results: To select natively folded HCV E2 mutant ectodomains, membrane thether chiemeras were evaluated for their stability and ER transport. HCV E2 412-423 epitope was stabilized by a disulphide bridge most likely in a beta hairpin conformation which was specifically recognized by AP33 antibody. The mutant HCV E2-6 was recombinantly produced and the differential binding of AP33-3/11 antibodies was confirmed. HCV E2-6 was more immunogenic comparing to wild-type and preliminary data showed that it induce higher nAb titers. The transmembrane HCV E1E2 heterodimer, HCV E2 and a soluble form of HCV E2 were transiently expressed in letuce, Nicotiana benthamiana and red algae *Porphyiridium*, respectively. Natively folded complex antigens were produced in all three expression systems and their immunogenicity and capacity to induce nAbs was proven using different routes of immunization (IV, oral or combined).

Conclusions: A workflow of vaccine antigen structure based design, recombinnat production and immunogenicity characterization was established. Its functionality was shown for HCV, HBV and it is currently extended to Marburg virus. Plant and microalgae complex proteins expression platforms were developed which may be used for other viral antigens or other biologicals production. The previously mentioned platforms will be completed by B-cell repertoire functional profiling for vaccine candidate prioritization or evaluation.

An oxidized tyrosinase derived epitope elicits an improved immune response

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Introduction: Melanoma is a cancer form which arises by malignant transformation of melanocytes- pigment producing cells. The main risk factor is prolonged UV light exposure, thus resulting in an increased incidence in recent years at global level. The discovery and approval of Immune Checkpoint Inhibitors (ICIs) has revolutionized the immunotherapy-based melanoma treatment. However, a subset of patients does not respond or develop resistance to immunotherapy and the overall survival rate is reduced. Clinical studies from recent years have demonstrated that combinatorial therapy has an increased effectiveness [1]. Thus, discovery of additional therapeutic agents such as neo/chemically modified antigens is of real interest.
One therapeutically exploitable hallmark of melanoma, as well as other cancers, is the display of specific or tumor-associated antigens on the cell surface, which can be utilized to design new immunotherapeutic targets. Among the specific melanoma antigens, tyrosinase is a tumor associated autoantigen presented via the MHC1 complex to the cell surface, nonameric sequences which can elicit an immune response. The most abundant is the tyrosinase$_{369-377}$-YMNGTMSQV sequence presented at the cell surface as naturally de-glycosylated YMDGTMSQ epitope, in varying amounts depending on the misfolding protein state and posttranslational modifications. Although *in vitro* stimulation of specific T cells with this peptide triggers a very good immune response, clinical trials of vaccines with the peptide alone or in combination with other antigenic peptides didn't reveal an improved clinical response. Here we address the antigenic potential of the oxidized derivatives of the tyrosinase epitope YMDGTMSQV. Since it is well known that methionine is an amino acid susceptible to oxidation under specific conditions, we were interested if oxidation of methionine residues from position 2 and 6 could induce a better immune response [1].

Materials and methods: The YMDGTMSQV peptide was oxidized under controlled conditions with hydrogen peroxide to obtain both the sulfoxide and sulfone forms of each or both amino acids. The kinetics of the oxidation reaction was determined by an analytical RP-HPLC method. A semi-preparative HPLC method was used to purify each oxidized form, and the identity and purity of the collected fractions was established by LC MS/MS and RP-HPLC analysis.
The immunogenicity of the purified fractions was verified by specific immunological assays: CTL lysis, IFNγ secretion and binding to specific HLA by a competition method. The possible intracellular production of oxidized forms was assessed by cell biology and mass spectrometry techniques.

Results: Using specific immunoassays and analytical methods such as mass spectrometry and RP-HPLC, we were able to show that oxidation of the methionine at position 6 of YMDGTMSQV triggers a better immune response, compared to the native peptide. Moreover, a small part of the oxidized fraction is produced intracellularly by melanoma cells[1].

Conclusions: Oxidation of methionine from specific immunogenic sequences elicits a better immune response in comparison to the non-oxidized counterpart.

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The transcriptional landscape of cancer stem-like cell functionality in breast cancer

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Introduction: Cancer stem-like cells (CSCs) have been extensively researched as the primary drivers of therapy resistance and tumor relapse in patients with breast cancer. However, due to lack of specific molecular markers, increased phenotypic plasticity and no clear clinicopathological features, the assessment of CSCs presence and functionality in solid tumors is challenging. While several potential markers, such as CD24/CD44, have been proposed, the extent to which they truly represent the stem cell potential of tumors or merely provide static snapshots is still a subject of controversy. Recent studies have highlighted the crucial role of the tumor microenvironment (TME) in influencing the CSC phenotype in breast cancer cells. The interplay between the tumor and TME induces significant changes in the cancer cell phenotype, leading to the acquisition of CSC characteristics, therapeutic resistance, and metastatic spread. Simultaneously, CSCs actively shape their microenvironment by evading immune surveillance and attracting stromal cells that support tumor progression.

Materials and methods: In this study, we associated in vitro mammosphere formation assays with bulk tumor microarray profiling and deconvolution algorithms to map CSC functionality and the microenvironmental landscape in a large cohort of 125 breast tumors.

Results: We found that the TME score was a significant factor associated with CSC functionality. CSC-rich tumors were characterized by an immune-suppressed TME, while tumors devoid of CSC potential exhibited high immune infiltration and activation of pathways involved in the immune response. Gene expression analysis revealed IFNG, CXCR5, CD40LG, TBX21 and IL2RG to be associated with the CSC phenotype and also displayed prognostic value for patients with breast cancer.

Conclusions: These results suggest that the characterization of CSCs content and functionality in tumors can be used as an attractive strategy to fine-tune treatments and guide clinical decisions to improve patients therapy response.

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A preliminary *in vivo* investigation of the effects of a combined immunotherapy on B16.F10 murine melanoma model

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Introduction: Immune checkpoint blockades (ICBs) have emerged as a key treatment for various types of cancers, showing encouraging results in difficult cases. ICBs target proteins involved in the crosstalk between cancer cells and immune cells, therefore blocking the cell death signaling inflicted upon immune cells. Melanoma is one of the best-suited candidates for ICB due to its high immunogenicity. However, tumor heterogeneity continues to limit success rates, indicating a need for therapy optimization. The aim of this study was to evaluate the efficacy of combined immunotherapy based on anti-PD-L1 antibodies and curcumin-loaded extracellular vesicles derived from stressed, peptide-pulsed dendritic cells in treating murine melanoma *in vivo*. This strategy exploits the efficacy of anti-PD-L1 therapies, the targeted delivery capability of extracellular vesicles, and the immunomodulatory properties of curcumin.

Materials and methods: A pilot *in vivo* study was conducted on C57BL/6 mice bearing B16.F10 melanoma tumors to assess the effects of anti-PD-L1 antibodies combined with EVs with curcumin (PEG-EV-CURC). The tumor volume and mouse body weight were monitored throughout the study. Post-treatment, tumors were excised, measured, and subjected to molecular analyses including the assessment of the level of expression of proteins involved in inflammation and apoptosis by western blot, of cytokines and growth factors involved in inflammation and angiogenesis by protein microarray, the activity of MMPs involved in invasion and metastasis by gelatin zymography, and the percentage of 5-methylcytosine, as a measured of epigenetic changes associated with tumor progression, determined by ELISA.

Results: The combined treatment led to a >50% inhibition of tumor growth compared to the growth of untreated tumors ($p < 0.0001$). Molecularly, the level of expression of critical proteins such as pAkt was significantly reduced (>50%), while the expression level of pNF-κB and iNOS, remained unchanged compared to control tumors. The combined therapy group exhibited a broad reduction in the expression of cytokines involved in inflammation and angiogenesis, including G-CSF ($p < 0.0001$), M-CSF ($p < 0.0001$), Eotaxin 1 ($p < 0.001$) and VEGF-A ($p < 0.01$), compared to the control group, with even more pronounced impact when compared to the group treated with anti-PD-L1 antibodies alone. As for the metastatic capacity, the MMP-2 expression levels were slightly elevated in the combined therapy cohort ($p<0.01$) compared to the control, while MMP-9 levels were not modified across all treatment groups. Furthermore, the combined therapy decreased cytosine methylation in tumors ($p < 0.01$) compared to the control group.

Conclusions: This combined immunotherapy shows potential in treating murine melanoma and may possibly be further optimized by adjusting the timing and dosage of administration of the encapsulated curcumin.

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Implementing an Undiagnosed Diseases Program in Romania: A Strategic Move to Address Rare Diseases

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For millions globally, living with a rare disease means facing uncertainty—no name, no diagnosis, and no targeted treatment. Rare diseases (RDs), each affecting fewer than 1 in 2,000 people in the EU, collectively impact around 350 million people worldwide. About 80% of these conditions are genetic, with 95% lacking approved treatments. This represents a major public health challenge.

Romania faces a critical need to address the burden of rare and undiagnosed diseases. Approximately 1.3 million Romanians suffer from rare diseases, yet 90% do not receive accurate diagnoses or appropriate care. This is even more pressing compared to the European Union, in which 40% of rare disease patients receive incorrect diagnoses. For Romania, innovative strategies are needed to bridge this gap and provide comprehensive care.

Inspired by the success of the National Institutes of Health's (NIH) Undiagnosed Diseases Program (UDP) and the creation of similar programs in Western Australia and throughout the world, Romania has the opportunity to establish its own UDP. The NIH UDP, launched in 2008, combines cutting-edge genomic technologies, multidisciplinary expertise, and a collaborative research network to solve complex medical cases. It has set a global standard for more than 20 countries and more than 50 institutions worldwide.

Adopting this model in Romania would provide several benefits:

1. Timely and Accurate Diagnoses. By leveraging advanced genomic technologies and multidisciplinary teams, Romania can improve diagnostic accuracy for rare and complex cases, reducing the delay in identifying conditions that impact patients' quality of life.
2. Personalised Medicine. A national program focused on undiagnosed diseases would enable personalised treatment plans tailored to the genetic and clinical profiles of patients, offering better management of symptoms and improved outcomes.
3. Integrated Care and Social Inclusion. Proper diagnosis and management of rare diseases would allow patients to access targeted care, enhancing their ability to participate fully in society and the workforce, thus reducing the overall socioeconomic burden.
4. Research and Innovation. Establishing a UDP would foster a collaborative environment for research, encouraging the development of new therapies and enhancing Romania's scientific contribution to rare disease research.

By replicating the UDP model, Romania can transform its approach to rare and undiagnosed diseases, ensuring better patient outcomes and setting a benchmark in Eastern Europe for integrated healthcare innovation.

Hit identification for the development of new therapeutic drugs for neurodegenerative diseases

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Introduction: - Neurodegenerative diseases represent a major health problem, due to their debilitating and incurable clinical manifestations. Targeting chronic neuroinflammation is regarded to bring promising therapeutic opportunities One of the molecular targets in inflammatory and neurodegeneration processes is the receptor for advanced glycation end products. RAGE and its canonical ligand, the neurotrophic factor S100B, were found to be overexpressed in Alzheimer's disease, Multiple Sclerosis, and Parkinson's disease, both in patients and in disease models. Studies indicate maladaptive roles of RAGE/S100B linked to an exacerbation of the neurodegenerative processes, and that inhibition of RAGE activity could decrease pathology and improve cognitive function [1]. In the present work, we aim at the identification of chemical scaffolds to interfere with receptor binding to S100B. We set up an *in vitro* method based on fluorescence detection to measure binding of S100B to RAGE, which was further used in a high throughput screen (HTS) of compounds.

Materials and methods: A time-resolved fluorescence resonance energy transfer (TR-FRET) assay was employed to measure RAGE-S100B interaction. The system comprised of a short variant of RAGE encompassing VC1 domains, with a sequence of six histidines -(His) tag at the C-terminus, and the FITC-labeled S100B. Proteins were obtained by recombinant DNA techniques, expressed in bacteria and purified by affinity chromatography. A Tb-labeled anti-His tag antibody acts as a FRET donor to the FITC-labeled S100B. 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 and for the HTS of an EU-OPENSCREEN chemical library (~100,000 compounds). Identified hits are further evaluated in functional assays in relevant cell cultures by immunofluorescence microscopy and Western blot assays. Molecular dynamic simulations and docking studies are performed to understand their mechanism of action.

Results: A TR-FRET assay with VC1-His and S100B-FITC was set up and optimized. 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. The performance of the assay was validated in a screen of a small library of natural products. The primary screen of the EU-OPENSCREEN library led to the identification of 875 hits. Hits were next tested in triplicates and in a counter assay with His-S100B-FITC. Dose response curves were further performed leading to the identification of 10 small molecules with a low micromolar IC50. Structural studies on the RAGE/S100B molecular models revealed the potential binding sites for the identified hits. Compound activity is further analyzed in cell cultures with endogenous RAGE expression, by quantification of S100B binding/internalization and subsequent cellular RAGE activation.

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

Acknowledgement: This work was supported by UEFISCDI grant PED159/2017 and EU-OPENSCREEN Small Molecules Screening Call (PID 9521/2019)

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Discovery of natural secretagogues of hormones that regulate glucose metabolism

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Introduction: One of the biggest challenges today in healthcare is overcoming the increasing trend in the worldwide prevalence of some severe diseases, such as type 2 diabetes mellitus and cardiovascular diseases. This trend is due to a significant change in eating behavior and a dramatic decrease in physical activities. Glucose concentration is regulated by insulin, glucagon and glucagon-like peptide 1 (GLP-1) and other peptide hormones. While current treatments in diabetes mellitus include exogenous insulin, GLP-1 analogues or GLP-1 receptor agonist, we aim to discover natural secretagogues of GLP-1, insulin and glucagon. Our strategy may have both preventive and curative effects.

Materials and methods: To uncover molecular mechanisms of insulin, glucagon and GLP-1 regulation and discover specific secretagogues, we engineered reporter cell lines introducing a luciferase within the sequence of each desired hormone in model cells. To study GLP-1 secretion we used GLUTag cells and 1.1B4 cell line for insulin secretion. Each newly-created reporter cell line recapitulates physiological regulation of GLP-1/insulin/glucagon, as demonstrated using western blotting and ELISA, together with specific, known secretagogues. A focused natural compounds library, as well as a library containing metabolites of gut microbiota was screened. To investigate the mechanisms by which newly found secretagogues potentiate insulin, GLP-1 or glucagon secretion we measured luminescence, glucose uptake and ATP concentrations.

Results: Upon posttranslational processing of the preproglucagon (or insulin) fusion, the cells release the specific hormone and luciferase stoichiometrically. Measuring luciferase activity is a lot faster and cheaper than quantifying hormones. Flavonoids, especially quercetin enhace secretion of GLP-1 (1), as well as insulin secretion. In GLUTag cells quercetin acts by increasing ATP concentration. We also found several gut microbiota metabolites that increase GLP-1 secretion.

Conclusions: We created a robust platform for the discovery of new compounds with anti-diabetic effects. Flavonoids are strong, natural GLP-1 and insulin secretagogues (1). However, the concentrations required to elicit an effect are physiological relevant for GLP-1, as the enteroendocrin cells that secrete GLP-1 are located throughout most of the gut, where flavonoids reach the highest levels in human organism.

Further studies are required to elucidate how gut microbiota metabolites can stimulate the GLP-1 secretion machinery, as well as their effect on insulin and glucagon secretion.

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BioMolViz: A Community Improving Education in Biomolecular Visualization

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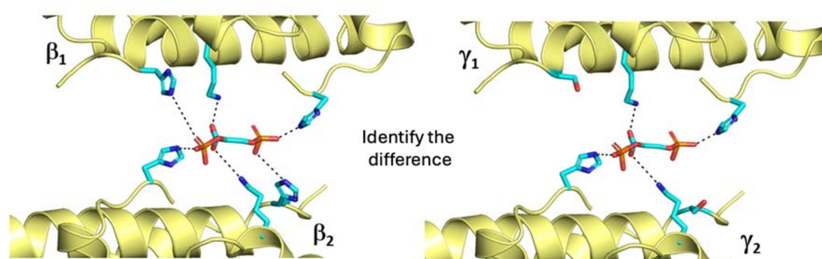
Introduction: To advance to expertise in biochemistry, students must understand and interpret images of biomolecules. This requires them to develop their visual literacy skills; however, explicit instruction on honing visualization skills is rare. Moreover, there is a lack of consensus in the community on how to both teach and assess biomolecular visual literacy [1]. To this end, BioMolViz, in collaboration with the biochemistry and molecular biology education community, developed a Framework that includes discrete learning objectives within overarching themes in biomolecular visualization [2]. Using this Framework for backward design, we convened faculty in workshops and online working groups to write and revise visual literacy assessments. Through a validation process involving expert panel review and classroom testing, we have evaluated our collaboratively designed assessments [3].

Materials and methods: For validation, we recruited four experts in both biochemistry and education to review assessments and rate them across 3 categories: clarity, relevance to the primary learning objective, and appropriateness for learner level. Assessments scoring a rating of 3.6 on a four-point Likert scale (1 = requiring major revisions to 4 = excellent, requiring no revision) were prepared for field testing. Two assessment sets were constructed, each containing at least 15 unique multiple-choice/multiple-select questions. Each assessment set was distributed using survey software across at least seven different institutions, and each survey of assessments received over 350 student responses.

Results: We examined student responses in several ways. Using classical test theory, we performed an item-wise analysis of difficulty and discrimination to identify which questions may be too challenging for the targeted learner level and which questions fail to distinguish between high- and low-performing students. Most (27 out of 31) of the assessment items met the criteria of difficulty indices in the range of 0.20–0.90 and discrimination indices ranging from 0.20–0.39. We flagged the 4 assessments outside of these ranges to be considered for further revision. We then examined all the assessment items for demographic-related performance differences & discovered 10 assessments showing gender-related disparities. Uncovering factors that contribute to the observed performance differences could guide more inclusive visual literacy assessment. Therefore, we have begun examining these trends through a group think-aloud protocol, where students discuss their thought processes while working through visual assessments presented by an instructor. We are currently coding the transcripts from these think-alouds to explore the cognitive processes students employ during visual problem solving.

Conclusions: We have collaboratively developed a set of validated, classroom-tested assessments that can be used to evaluate student visual literacy skills. We have begun developing methods that utilize these assessments to understand the cognitive processes that students use during visual problem solving. We encourage educators to incorporate image-based instruction and assessment into their courses to ensure that biomolecular visual literacy becomes an integral part of the learning process in the life sciences.



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The Dawn of the Adaptive Immune System. Advent and Evolution of the RAG System

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We celebrate these days the 25th anniversary of the Department of Bioinformatics, established in 1999. Ever since, combining in-silico methodologies with experiment has helped increase the pace of research in Molecular Medicine, Glycobiology, Immunobiology, Pharmacology, Systems Biology and many other fields of Molecular Life Sciences. Among these a major focus of our group over the past decade was on the evolution and structure function relations of the Recombination-activating gene products RAG1/2 responsible for the V(D)J recombination found at the heart of the adaptive immune system. In early stages of this endeavor, starting from very remote homologues we were able to model the central RnaH active region of RAG1 [1], the 6 blade propeller region of RAG2 and using FRET constraints the tight bend of the DNA Recombinant Signal Sequences RSS12/23 [2,3] - all confirmed several years later by the first tetramer RAG complex crystal structures.

Given that the RnaH active site of RAG1 is also found in all DDE transposases and integrases it was long presumed that the RAG system has evolved from transposons and indeed a first protoRAG transposon was discovered in the deuterostome lancelet Branchiostoma belcheri in 2016. Our experience in remote homology modeling of RAG system allowed us building the model used to fit the B.belcheri protoRAG Cryo EM data [4]. Solving the protoRAG structure was key in understanding the mechanism and identifying the sequence characteristics modulating transposition. This bought critical insights into the relation between transposition and recombination as aberrant RAG induced transposition is known to promote lymphocyte malignancies and various types of genomic instability.

But how spread and diverse such RAG ancestors are in the animalia kingdom. Using an intricate sequence profiling analysis we were able to iteratively find more and more members of various protoRAG types in increasingly distant species going further down into the protostome clade and even cnidaria suggesting an ancient bilaterian origin of protoRAGs [5]. This pushed the advent of RAG system with at least 350 Million years earlier than previously thought form the chordate model.

A critical problem that still remains open is on how and when protoRAG domestication took place and the recombinase specific signatures first occurred into the protoRAG transposase frame. In this realm too our in-silico techniques have brought recently some answers by the discovery in some Hemichordata and Echinodermata of a new transposon group, the protoRAG family A which show incipient recombinase traits [6]. Currently we aim to deepening our understanding of the transition from transposition to recombination and of the mechanisms allowing suppression of transposition in chordate RAG systems.

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Exploring the glycoproteomic space of the early secretory pathway in melanoma cells with disrupted ER homeostasis

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Introduction: The secretory pathway is a key-component of the mammalian cells in regulating their homeostasis. This is reflected by various pathologies which involve dysfunctional proteins often designated as misfolded proteins. A great fraction of these is N-glycosylated and involves the assistance of specific chaperones to attain their native conformation. The cell needs to eliminate them and send only the correctly folded fraction for intracellular sorting or extracellular export, often involving an intricate network of mannosidases with dual roles in glycoprotein degradation and maturation. Studies, using mostly canonical misfolded proteins, have shown that ER degradation-enhancing alpha-mannosidase-like protein 2 (EDEM2) is the first mannosidase required in this sorting process [1].

Materials and methods: Using liquid chromatography coupled with high resolution mass spectrometry we compared the N-glycoproteome of cells with up-regulated or down-regulated EDEM2 expression. The proteins extracted from ctr or treated cells were subjected to protease digestion and the obtained peptides were further used for lectin-based enrichement of oligomanosidic glycans. These were released using Endo H and the resulting partial deglycosylated peptides were further analyzed by nanoLC-MS/MS. The same methodology was also used for the N-glycoproteome analysis of cells treated with kifunensine, a class I mannosidase inhibitor.

Results: Here, using a dedicated glycoproteomics workflow we find novel potential clients of EDEM2 in A375 melanoma cells, among them a truncated version of tyrosinase melanoma autoantigen, protocadherin 2 or integrin alpha 1 [2]. We confirm some of these by proteome-wide measurements of protein kinetics and by cellular and biochemical methodologies. Moreover, we extend our glycoproteomic approach to differential analysis of the oligomannosidic glycans released from N-glycosylated proteins and find out that protein core-fucosylation is predominantly downregulated in cells with inhibited activity of class I mannosidases [3].

Conclusions: Our results emphasize the regulatory role of EDEM2 in glycoprotein degradation and trafficking modulation, particularly impacting tumor antigens and proteins associated with melanoma metastasis. Moreover, using system-wide -omics technologies, we dissect the steady-state oligomannosidic glycoprotein load in A375 melanoma cells and the role of class I mannosidases in the intracellular sorting process for some of these. Our results suggest a vital connection between ER homeostasis and cancer biology.

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Robosample: A Robot Mechanics Molecular Simulation Software for Enhanced Exploration of Biomolecular Conformational Dynamics

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Introduction: Biological processes are typically cascades of conformational transitions and interactions of macromolecules. Switch-like conformational shifts are rare events that involve transitioning from one potential energy well to another which is often hindered by the need to overcome a high energy barrier or the navigation of elusive pathways between the wells. This makes describing these transitions using methods such as Molecular Dynamics (MD) or Markov Chain Monte Carlo (MCMC) extremely challenging.

The need to investigate transition pathways in molecular biological processes has driven the development of numerous enhanced sampling methods, including those that use modified Hamiltonians attempting to find a better reaction coordinate. Here, we introduce Robosample [2], a software package that features the ability to design Hamiltonians based on sensitive molecular regions, leveraging generalized coordinates from robotic mechanics to study large configurational changes.

Materials and Results: Robosample encodes molecules in multibody systems articulated by joints - aka robots - and modifies their configurations using generalized coordinates. The simulations are conducted using the GCHMC method (Hamiltonian Monte Carlo coupled with Gibbs sampling [1]) which essentially takes turns in sampling different degrees of freedom of the molecule.

Besides the use of generalized coordinates, including Cartesians or more natural Bond-Angle-Torsion coordinates, articulated multibody representation facilitates straightforward access to generalized velocities and torques, and enables the calculation of their associated statistics. Consequently, statistical analysis is employed to identify the most sensitive regions of the macromolecule, which aids in refining the initial guess of the reaction coordinate. The analysis considers both the instantaneous generalized tensions, derived from the Jacobian associated with coordinate transformations, readily accessible through robotic mechanics algorithms, as well as the fluctuations that reveal the type of pressure exerted on specific molecular bonds. In the current work we demonstrate the utility of this type of analysis on a series of simulations of a diverse set of proteins obtained randomly from CATH database.

Conclusions: The results indicate that early identification of sensitive regions can more effectively guide constrained simulations for analyzing transition pathways.

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Longevity Genie: Enhancing Large Language Models for Longevity and Genetics Research through Retrieval-Augmented Generation and Specialized AI Agents

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Introduction: Large Language Models (LLMs) have shown limitations in biomedical research, particularly in longevity and genetics, due to lack of specialized knowledge, poor referencing, and tendency to hallucinate. The Longevity Genie project aims to address these challenges by augmenting LLMs with domain-specific knowledge and advanced retrieval techniques.

Methods: We developed the Longevity Genie system, integrating Retrieval-Augmented Generation (RAG), hybrid search techniques, and AI agent workflows. This approach incorporates domain-specific databases and utilizes specialized agents for precise querying and structuring of scientific information. We also explored the integration of biomedical knowledge graphs to improve inter-domain reasoning.

Results: Our project has yielded several functional tools:

1. A Genetics Genie custom assistant (https://chat.longevity-genie.info/) supporting multiple models for genetics and clinical trials research.
2. Custom GPTs including Genetics Genie GPT (https://chat.openai.com/g/g-HnvTUyFeq-genetics-genie), Clinical-Trials Genie (https://chat.openai.com/g/g-tpOIOEeWm-clinical-trials-genie), DrugAge Genie, and AnAge Genie (https://chat.openai.com/g/g-I8OGEVL4X-animal-ageing-genie).
3. The Longevity GPT (https://asklongevitygpt.com/) implementing RAG and advanced referencing techniques.
4. Open-source libraries for agent organization (just-agents: https://github.com/longevity-genie/just-agents/) and research paper handling (getpaper, indexpaper).
5. Aging-related papers datasets hosted on Hugging Face (https://huggingface.co/longevity-genie) to support further research.

These tools demonstrate improved performance in genetics interpretation, clinical trial information retrieval, and longevity-related query handling.

Conclusions: The Longevity Genie project successfully addresses key limitations of LLMs in biomedical and longevity research. By integrating domain-specific knowledge and advanced retrieval techniques, we have created a suite of tools that enhance the accuracy and reliability of AI-assisted research in these fields. This approach contributes to democratizing access to advanced AI tools in biomedical research, potentially accelerating progress in longevity studies and related fields. Future work will focus on further improving the system's capabilities and expanding its knowledge base.

Joints in Motion: Accelerating Protein-Ligand Insights with Robosample

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Introduction: Molecular simulations are essential to get atomic-level insight into biological molecules' behavior and interactions, yet they remain computationally intensive especially for estimating probability distribution based observables such as drug-macromolecule binding affinities. To address this challenge, we have previously developed Robosample [2] molecular simulation software that combines robot mechanics and the enhanced sampling method GCHMC [1]. Here, we expand the software's methodology by incorporating automatic determination of Gibbs blocks and introducing essential logical and hardware innovations that substantially improve its accuracy in predicting protein conformations and ligand-protein interactions.

Materials and methods: Robosample represents molecules as rigid bodies connected by joints, where the rigid bodies are sets of constraints, joints are Gibbs blocks and the types of joints are functions of the relative Cartesian coordinates. The number of rigid bodies allows for a fine- to coarse grained approach, ranging from individual atoms to a fully rigid molecule. We introduce a bottom up bond-angle-torsion (BAT) molecular graph representation that allows for easy choosing of rigid body constraints across the Gibbs blocks. Also, BAT representation allows for easy exchange between types of joints, significantly enhancing simulation efficiency. While previous versions were limited to small molecules and glycans, this new representation allows for the accurate simulation of large macromolecules, expanding the applicability of our software to a wider range of biological systems. The main software optimizations included full utilization of LAPACK for linear algebra computations, compilation of all dependencies into a single executable, binary layout optimization for increased instruction cache hits, and externalization of Cartesian type-joints to a high-speed integration library, resulting in a 40-fold improvement in execution speed. The entire functionality is exposed through a Python interface enabling integration with external molecular modeling tools.

Results: To validate the correctness of conformational space sampling, we simulated a set of 10 diverse proteins from the CATH database and compared them to their NMR-resolved structures. The results demonstrate that our software can accurately reproduce the experimental protein conformation distribution. Next, we used it to predict binding sites and poses for ligands interacting with RAGE (Receptor for Advanced Glycation End-products). Moreover, we are able to detect internal tensions in generalized coordinates, which are crucial for both molecular modeling, protein engineering, and understanding conformational changes.

Conclusions: These advancements elevate our software into a powerful tool for drug screening, design studies, and protein engineering, where computational efficiency and predictive power are crucial. By enabling accurate simulations of entire proteins and enhancing our ability to predict ligand-protein interactions, our software significantly broadens its applicability and provides deeper insights into molecular dynamics and structural biology.

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Prediction of NOD like receptor structure with new, state of the art Deep Learning methods. Deep Learning, ma non troppo

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Introduction: Artificial Intelligence and Deep Learning are among the fastest growing scientific fields in the current data reach technological age. It is largely accepted that Structural Biology has also entered a new era with the advent of these deep learning techniques implemented in the new generation of predictors that include AlphaFold and RosettaFold. In the current work we aim to evaluate the prediction capabilities of these new DL methods on more complex multidomain proteins rather than simple, single domain ones. To this end we used three of the most well-known platforms: Alphafold2, Alphafold3 and RosettaFold-All-Atom in order to generate over 1000 protein models of a Coiled-coil family of NOD-Like Receptors (CNL) from Arabidopsis thaliana on which we have gained extensive structural insight over the past decade [1,2,3]. CNLs are multidomain proteins consisting of three main domains CC, NBD and LRR. Of these NBD further contains three subdomains: NBS, Arc1 and Arc2 - giving a total of 5 modules that, upon activation, change both their relative configuration and their bonded ligand from ADP in the inactive state to ATP in the active state. CNL models were then examined with respect to their intrinsic quality and to the current experimental data in order to better understand DL method limitations.

Materials and methods: Based on clustering at 70% coverage and 70% identity of the entire A.thaliana CNL set consisting of 1257 sequences in NLRscape database [2], followed by the examination of domain structure integrity and presence of all CNL motifs identified with NLRexpress [3] a clean representative subset of 33 sequences was selected. Models of these 33 sequences were generated with Alphafold2 installed on IBAR computing cluster and modified in-house to use a selective subset of structural templates for structure generation and a subset of sequences for the MSA block of the neural network. The templates were NLR proteins present on the RCSB, in either active or inactive form, as well as a set of proteins belonging to the 1.20 (Up-Down Bundle) and 3.80.10 (Leucine-Rich Repeat) CATH families. The sequences used in the MSA were proteins of the NLR family. As a positive control, we also ran an Alphafold2 job using no MSA, only feeding structural templates into the network. In addition for all the 33 selected sequences we have downloaded Alphafold2 Database models and also generated them in interaction with ADP (inactive state) and ATP (active state) using Alphafold3 and RosettaFold-All-Atom.

Results: Analysis indicate that DL techniques are able to predict in quite good agreement the structure of the individual CNL modules especially the LRR domain and the three NBD subdomains but show significant bias toward the inactive form in modeling the overall CNL configuration, even in the presence of ATP. Alphafold models are closer to expectation than the Rosetta ones and generally display higher quality scores. Even after model optimization some of the Rosetta models fail quality tests and show significant departures from expected module structure.

Conclusions: Results suggest that the new generation of DL methods give swiftly good results for single domains especially when closer templates are at hand. However while AI-driven modelling has speed-up structure generation and removed much of the tedious work involved in euristic manual modeling - AI 3D models of more complex protein sequences or of sequences lacking close templates might show departures from the real global architecture and should be taken with care.

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Antimicrobial efficacy of novel cell-penetrating peptides

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Introduction: Treatments for cancer, neurological problems, and viral infections are necessary due to the demands of modern lifestyles and longer life expectancy [1,2]. Therefore, there is a requirement for novel, efficient, and reliable techniques that provide a high level of specificity in targeting cells, are economically viable, and have little effects on healthy tissues. Peptide-based systems provide the capacity to transport medicines and genes. Cell-penetrating peptides (CPPs) are compounds that may effectively cross cell plasma membranes to deliver various substances, including drugs, genetic material, and imaging agents, into the cytosol or nucleus. They exhibit chemical versatility, stability, and non-immunogenicity.

Materials and methods: Novel CPPs were synthesised using solid-phase peptide synthesis (SPPS) technique, employing Fmoc chemistry and a solid Rink-amide resin, on an automated peptide synthesiser. There are five peptides with a free amino terminus, and five peptides with a stearyl moiety group attached at the N-terminus. The peptides were isolated by preparative high-performance liquid chromatography (HPLC). The purity of the samples was assessed using analytical high-performance liquid chromatography (HPLC), and their identification was verified using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) or TOF-MS-MS. The antibacterial effectiveness of the peptides against *Escherichia coli, Pseudomonas aeruginosa, Salmonella typhimurium, Staphylococcus aureus, Bacillus subtilis* subsp. *spizizenii* was assessed using the broth microdilution method, with the Minimum Inhibitory Concentration (MIC) values serving as the evaluation criteria. The toxicity, growth rate, and metabolic activity of cells were assessed using microscopy, the Alamar Blue assay, and/or flow cytometry at various doses of the peptide on cancer and normal cells.

Results: Ten peptides were obtained and characterized. The antimicrobial activity (against tested bacteria) is depended on the peptides' sequence and peptides concentration. The majority of the peptides presented a concentration dependent toxicity in breast cancer cell lines - MDA-MB-231 and MCF-7, multiple myeloma cell line - U266B1, and human T lymphocyte cells - Jurkat cells. The peptides do not exhibit toxicity against mesenchymal stem cells, however they do demonstrate toxicity at high concentrations towards primary peripheral blood mononuclear cells (PBMC).

Conclusions: In order to rapidly progress peptide-based therapeutic systems, it is crucial to develop peptides that can effectively enter the cell membrane or overcome biological barriers. These peptides should also have long-lasting presence in the body, while being safe and without causing immune reactions in humans.

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Cellular responses to ER stress in the absence of EDEM proteins from a multicellular organism

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Introduction: Misfolded or incompletely folded proteins are recognized by the ER quality control surveillance system and removed from ER to be degraded by the proteasome in a process called ER associated degradation (ERAD). Dysfunctions in ERAD lead to ER stress by overload with defective proteins, which further disrupts ER homeostasis. In humans, ER stress has been associated with a wide range of pathologies such as age-related maladies, cancer, metabolic diseases and inflammation. We used *Caenorhabditis elegans* to study the cellular responses to ER stress induced by disruption of ERAD, in the context of a whole, multicellular, organism.

Materials and methods: *Strains and genetic methods.* WT and loss of function alelle of *edem-1*, *edem-2, edem-3, ire-1, xbp-1, pek-1,* and *atf-6* mutants were cultivated at 20° C, otherwise indicated and fed with *E. coli* OP50 . Double, triple and quadruple mutants were obtained by standard genetic crosses. Gene silencing was performed by RNAi feeding.
Transgenic nematodes. To produce transcriptional reporters, 2.2 kb DNA sequence located immediately upstream of *edem-1*, *edem-2* and *edem-3* was cloned into pPD95.75 vector, respectively. To obtain translational reporters, a genomic DNA sequence consisting of about 2.2 kb of the promoter, first exon, first intron, and the rest of cDNA was fused in frame to mCherry or GFP, respectively. To obtain transgenic nematodes, the transgene was coinjected pRF4 vector that expresses the rol-6(su1006) dominant marker.
Microscopy. Images were acquired using a Zeiss LSM 710 laser scanning confocal microscope using identical settings for each reporter.
ER stress was induced by overexpression of CPL-1* misfolded protein or by treatment with tunicamycin, kifunensine and heat shock.

Results: As in mammals, *C. elegans* has three EDEM proteins, EDEM-1, EDEM-2 and EDEM-3, that have an evolutionarily conserved role in disposal of misfolded proteins through ERAD and thus, reducing ER stress. Although the expression of each *edem* is detected in specific tissues under physiological conditions, their expression is upregulated in additional cells upon ER stress. *edem-2* mutants show phenotypic pleiotropy under physiological conditions that are not enhanced by the loss of EDEM-1 or EDEM-3. Under ER stress conditions, *edem-2* and *edem-3* mutants exhibited reduced basal expression of both *xbp-1s* and GRP78/BiP homologue, *hsp-4*, and mitigated their expression upon ER stress, in association with increased resistance and survival.

Conclusions: (1) We report the first characterization of combined roles for *edem-1*, *edem-2* and *edem-3* functions under physiological and ER stress conditions in *C. elegans.* (2) EDEM-2 has a major role in the clearance of misfolded proteins from ER under physiological conditions, whereas EDEM-1 and EDEM-3 roles become evident under acute ER stress. (3) Preconditioning to EDEM loss activates a hormetic XBP-1 independent adaptive program that enhances ER stress proteotoxic responses to promote organism survival under acute ER stress.

Extracellular vesicles: friends and foes in cancer treatment

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Our latest research has focused on both facets of extracellular vesicles (EVs) in the tumor microenvironment (TME) as intercellular communication tools that promote tumor progression as well as on their potential to be used as drug delivery systems [1-4]. EVs shape the TME through complex interactions between malignant and stromal cells, facilitating the bidirectional transfer of functional molecules and significantly enhancing tumor development [1]. Thus, our earlier studies on tumor-derived EVs (TEVs) demonstrated that EVs induced by doxorubicin (DOX) administration provided a protective effect to hypoxic C26 colon carcinoma cells and stromal cells, reducing their responsiveness to DOX and further contributing to chemoresistance in colorectal cancer [2]. Beyond revolutionizing our understanding of cancer cell-stromal cell crosstalk in the TME, EVs' ability to selectively transfer cargo to cells underscores their potential for tumor-targeted treatment approaches [1,3,4]. Thus, our recent study optimized melanoma cell-derived EVs as drug delivery systems for DOX. After TEVs purification by ultrafiltration followed by size-exclusion chromatography, they were stabilized with poly(ethylene glycol) (PEG) for *in vivo* administration. Notably, PEG-coated TEVs encapsulating DOX (PEG-EV-DOX) have shown superior inhibition of melanoma growth compared to that exerted by clinically applied DOX liposomal formulation [3]. Furthermore, a novel combination therapy involving PEG-EV-DOX and simvastatin-loaded, IL-13-functionalized liposomes (IL-13-LCL-SIM) showed potent antitumor effects by disrupting the molecular communication between tumor-associated macrophages (TAMs) and melanoma cells [4].

Lately, we have expanded the potential of EVs as drug delivery systems to include those derived from stromal cells, such as EVs secreted by activated dendritic cells, which will be tested for their ability to enhance the efficacy of anti-PD-L1 immunotherapy in melanoma.

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Targeting unconventional protein secretion in inflammatory disorders

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Introduction: The innate immune response recognizes invading pathogens or noxious substances and initiates signaling cascades to coordinate an inflammatory response. An immediate response is the assembly of inflammasomes, multi-protein signaling complexes that control the release of cytokines and pro-inflammatory molecules. Whilst inflammasomes are critical for anti-microbial immunity, uncontrolled inflammasome activation leads to pathological inflammation in many human diseases, such as atherosclerosis, septic shock syndrome, neuroinflammation, post-stroke cardiac dysfunction and cancer, among others [1,2]. **Interleukin (IL)-1β** is the most potent pro-inflammatory cytokine synthesized as inactive form (proIL-1β), proteolytically cleaved by inflammasome-activated caspase-1 to its mature form (mIL-1β) and rapidly secreted to the extracellular space. IL-1β along many other pro-inflammatory cytokines/proteins are exported by an endoplasmic reticulum (ER)/Golgi-independent pathway, commonly referred to as **unconventional protein secretion (UPS).** One common factor required for the secretion of unconventionally secreted cargoes, both in mammals as well as lower Eukaryotes, are the peripheral Golgi proteins **GRASP55/65.** Although discovered as Golgi stacking proteins, later studies shown these proteins play key role in unconventional protein secretion both in physiological and pathological conditions [3].

Materials and methods: *CRISPR/Cas9 genome editing* was designed to add a small tag to the C-terminus of the IL-1β-encoding gene at its genetic locus which allows its detection under stimulated conditions. PCR genotyping was done with the Mouse direct PCR kit. *Secretion assay and luminescence detection:* Cells were stimulated as described previously [3] and the luminescence corresponding to secreted IL-1β was detected using the Nano-Glo® HiBiT Extracellular Detection System, following the manufacturer's instructions. *ELISA:* Secreted IL-1β in cell culture supernatant and mouse serum was determined using Mouse IL-1β/IL-1F2 DuoSet ELISAELISA from R&D Systems according to the manufacturer instructions.
Western blotting: Cell extracts and corresponding media was processed as described previously [3] to detect IL-1β expression level after cell exposure to inflammasome-activation stimuli. *Immunofluorescence microscopy:* protein co-localization was done as described before [3].

Results: Designing an IL-1β reporter cell line is challenging due to the complex process of generating the biologically active protein and classical overexpression systems would induce a constitutive inflammatory response which does not maintain the physiological context. Therefore, we aimed to generate a **CRISPR/Cas9 knock-in-IL-1β** reporter cell line, which recapitulates the physiological response to classical inflammatory stimuli and can be used for quantitative assessment of IL-1β secretion. We validated the obtained cell line for its capacity to actively secrete tagged IL-1β upon exposure to inflammatory stimuli and used it to screen an FDA approved drug library. Our objective was to identify already approved drugs which could be repurposed to anti-inflammatory medicines by blocking IL-1β release from innate immune cells. We identified 3 drugs which efficiently inhibit IL-1β secretion, validated them in primary macrophages and used the best candidate for final validation in an animal model for sepsis. As our previous results showed GRASP55 deletion leads to an impaired IL-1β secretion [3], we tested, using primary macrophages isolated from GRASP55$^{-/-}$ mice, whether the molecular target of these drugs is downstream GRASP55 or they have an additive effect, thus targeting a different export pathway.
Conclusions: We generated an endogenously tagged-IL-1β reporter cell line that was successfully used to screen a library of FDA approved drugs, validated the top 4 molecules in primary WT and GRASP55$^{-/-}$macrophages and tested the best candidate in a mouse model for its potency to reduce LPS-induced septic response.

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The cellular and molecular landscape behind ELK3-triggered metastasis in triple-negative breast cancer

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Introduction: Metastasis remains the primary cause of death in breast cancer, lacking targeted therapies. Unraveling the molecular mechanisms driving this process is crucial for developing treatments aimed at metastatic tumor cells. Therefore, the aim of this study was to investigate the involvement of the ELK3 transcription factor (TF) in triple negative breast cancer (TNBC) metastasis, by identifying the metastasis-related cellular and molecular processes affected by it.

Materials and methods: MDA231 TNBC cell lines in which ELK3 is either overexpressed (OE) or knocked-down (KD) were generated by a lentivirus-assisted cell transduction procedure. Genetic modifications were confirmed by flow cytometry via GFP+, and by both PCR and RT-qPCR. Whole transcriptome analysis of ELK3-KD cells was performed by microarray, in order to identify the genes modulated by this TF. Validation of the microarray data was performed by RT-qPCR. Alterations of three metastasis-related cellular characteristics predicted to be modulated by ELK3 based on the molecular data in Ingenuity Pathway Analysis software (IPA) were further validated *in vitro*, in both ELK-OE and ELK-KD cells. The migration capacity was monitored using 3D microfluidic devices, the stemness potential was assessed through mammosphere formation assays, whereass proliferation was evaluated by the AlamarBlue assay.

Results: More then 95% of all the lentiviral transduced cells were genetically modified after cell selection, for both ELK-OE and ELK-KD cells. The microarray analysis on ELK3-KD cells revealed ELK3-modulated transcriptome alterations for 765 genes. Based on this molecular signature, two major cathegories of cellular processes were predicted to be affected: cell migration [upregulated motor dysfunction / downregulated cell movement, invasion and organization of actin]; stemness capacity [upregulated cell cycle progression / downregulated self-renewal capacity]. RT-qPCR carried out on a set of ten genes pointed out *HIF1a, FGFR1, NDGR1, BNIP3L, DUSP1* and *PLAUR* as key modulators of ELK3-dependent processes. At the cellular level, modulation of ELK3 significantly affected cell migration in 3D microfluidic devices: its overexpression boosted migration speed (+31.2%) and persistence (+38%), while its downregulation inhibited cell migration (-22.1% for speed and -23.4% for persistence). ELK3 expression also impacted the stemness potential of the cultured cells, as its upregulation increased both spheroid size (+17.8%) and number (+49.2%) in mammosphere formation assays. Furthermore, cell proliferation was inhibited in ELK3 overexpressing cells (-22% at 24h), while ELK3-KD stimulated cell growth and division (+29% at 24h).

Conclusions: ELK3 is a pro-metastatic TF in TNBC, stimulating both 3D confined cell migration and the acquisition of the stem-like phenotype, while hampering cell proliferation. If these data are confirmed in *in vivo* studies and under clinical settings, ELK3 might become a molecular target in a TNBC migrastatic therapy.

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A transcriptomic analysis of the efficacy of the anti PD-L1 antibodies-based immunotherapy co-administered with dendritic cells-derived extracellular vesicles loaded with curcumin in B16.F10 murine melanoma

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Introduction: Current understandings on immunotherapies in cancer have led to promising discoveries along with a better quality of life. So far, immune checkpoint inhibitors such as Anti PD/PD-L1, CTLA-4 managed to overcome the challenges of treating melanoma, having promising results, while having minimal side effects, as is the case with classical therapies. However, advanced stage melanoma can become refractory and acquire resistance. In this regard we sought to create an adjuvant to immunotherapy based on extracellular vesicles purified from activated dendritic cells and loaded with curcumin, to potentiate its effect.

Materials and methods: To this aim, the combined therapy was tested on a three-dimensional model formed of B16.F10 murine melanoma cells, dendritic cells 2.4 and $CD8^+$ murine lymphocytes and further melanoma-bearing mice. The extracellular vesicles produced by dendritic cells 2.4 activated with CpG and pulsed with TRP-2 peptide were purified by ultrafiltration- size exclusion chromatography, the size was validated by DLS analysis and were further characterized by Western blot as shown previously (1)(2). RNA-seq analysis was performed to obtain an overview of the changes induced over the expression of genes involved in different protumor processes, followed by RT-qPCR, protein array to validate transcriptomic results.

Results: The results show that the extracellular vesicles loaded with curcumin co-administered with anti-PD-L1 displayed a higher antitumor activity compared to the administration of each treatment alone reducing the viability of the cells inside the spheroid by two-fold ($p < 0.0001$). Our transcriptomics data have shown that the combined treatment had antiproliferative and anti-angiogenic effects, affecting *de novo* formation of the lymphatic and blood vessels and tumor metabolism.

Conclusions: In conclusion, the curcumin-loaded dendritic cell-derived extracellular vesicles might sensitize melanoma tumors to immunotherapy based on anti-PD-L1.

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Simvastatin effect on lipid metabolism of normoxic B16F10 murine melanoma cells

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Introduction: As a competitive inhibitor of the rate-limiting enzyme of cholesterol biosynthesis, 3-hydroxy-3-methylglutaryl-Coenzyme A reductase, (HMG-CR), simvastatin (SIM) has demonstrated antitumor effects at high doses [1-3]. Moreover, our previous study has proved that under normoxic conditions, the expression of hypoxia inducible factor 1 (HIF-1) - a central regulator of cancer cell metabolism, was inhibited by SIM [2-3].

Materials and methods: As a follow-up of our earlier findings, in this study we explored the impact of SIM at IC50 on the lipid metabolism of B16.F10 murine melanoma cells, under normoxic conditions. In close connection with SIM's primary pharmacological role, we explored the lipophilic statin effect on the cholesterol biosynthesis as well as fatty acid metabolism. Thus, RNA-seq analysis for mRNA expression was performed, while western blot analysis was used to assess the levels of the key proteins such as, HIF-1α as metabolic regulator as well as proteins involved in lipid metabolism pathways (fatty acid synthase (FASN), stearyl-CoA desaturase 1 (SCD1), and acyl-Coenzyme A oxidase (ACOX3)).

Results: Our data suggested that SIM significantly modulated the expression of genes encoding for multiple proteins involved in lipid metabolism.

Conclusions: These data suggest that SIM caused a transition toward unsaturated fatty acid metabolism and isoprenoid production favoring melanoma cell proliferation and metastasis.

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Nanotechnology-based approaches in biomedical applications

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Introduction: Antimicrobial resistance represents one of the most serious threats to human health and new drugs are urgently needed. Unfortunately, the dramatic worldwide rise of multidrug-resistant bacterial pathogens cannot be counteracted by the current low pace of developing novel therapeutics with new modes of action. Nanotechnology together with our increase knowledge on infectious diseases has promoted significant developments in the field of antimicrobial drug delivery and medical implants. Here we present our main results regarding different types of nanoparticles and surface functionalization strategies that have demonstrated *in vitro* antimicrobial activity against pathogenic bacteria (*Enterococcus faecalis, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa*, *Enterobacter* sp., *Mycobacterium terrae*) and fungi *(Candida albicans)*. The results represent promising research directions towards a better control of nosocomial infections and microbial biofilms.

Materials and methods: Different nanostructures (graphene oxide [1], hydroxyapatite [2], iron oxide [3]) were investigated for their potential to improve the antimicrobial properties of different natural or synthetic compounds (isoniazid, pyrazine-2, carbohydrazide [1], plant essential oils [2], fluorenones derivatives [4], thiadiazolo[3,2-a] pyrimidine derivatives [3], thiourea derivates [5]). Broth-microdilution and microtiter plate methods were used to assess biological activity. Potential mechanisms of antimicrobial activity were investigated using flow cytometry and live-dead fluorescence microcopy. Human mesenchymal, Hela and HEp-2 cells were used to evaluate the biocompatibility of the compounds.

Results: The nanostructures exhibited promising anti-adherence properties and significantly improved the bioactivity against planktonic microbial cells, by the incorporation of the compounds/plant essential oils. The microbicidal effects of the hybrid materials based on the covalent grafting of isoniazid and respectively, of pyrazine-2-carbohydrazide to graphene oxide were associated with the induction of bacterial membrane depolarization. The tested hybrid materials exhibited low cytotoxicity levels.

Conclusions: Our results demonstrated that the incorporation of the tested compounds into different types of nanostructures induced an enhanced anti-biofilm activity. Moreover, graphene oxide alone exhibited a very good intrinsic anti-mycobacterial activity which was increased when coupled with isoniazid. Thus, nanotechnology advances new avenues for the treatment of infectious diseases caused by drug-resistant pathogens.

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First molecular investigations using laser-induced high-energy electron FLASH irradiation to characterize cancer cells response to ultrahigh dose rate exposure

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Introduction: Radiotherapy has been one of the key options of treatment in the oncologist's toolkit since the radiation was first discovered at the end of 19th century. Cancer cells exposed to radiation die as a result of irreparable DNA damage. However, upon repeated fractionated regimes, some tumors develop resistance to radiotherapy; also, the normal tissues surrounding the tumors are often affected by radiation-inflicted toxicity. Recently, new radiation regimes have been explored such as those emitted at ultrahigh dose rates (UHDRs), which have the potential to be used as a new anti-cancer therapeutic strategy. The FLASH effect induced by UHDR irradiation has been shown to maintain anti-tumor efficacy in animal models, while sparring the functionality of normal tissue [1]. Here, we present our recent studies aimed to demonstrate this effect *in vitro* for very high energy electron (VHEE) irradiation [2] with a view to increase the understanding of the effect of this type of treatment at cellular and molecular level.

Materials and methods: We used the high-intensity petawatt (PW) laser-driven accelerator in CETAL facility that can deliver VHEEs at dose rates as high as 10^{13} Gy/s in very short pulses (10^{-13} s). A375 melanoma cancer cells and normal non-transformed melanocytes were concurrently exposed to laser-plasma accelerated (LPA) electrons. In parallel, co-cultures were exposed to X-ray – the standard DNA damage producing source. Phospho-gammaH2AX positive cells and nuclear foci were quantify as a measure of radiation-induced DNA damage using immunofluorescence and TissueFAXSiPlus imaging system. Cisplatin served as chemical control for assay set-up.

Results: A non-uniform FLASH dose distribution over the SlideFlask surface supporting the cell co-culture was revealed by Gafchromic films placed behind the recipient vessel. The dose gradient was consistent with the nuclei containing damaged DNA 30 minutes post-exposure. The highest proportion of the p-gammaH2AX^{+} nuclei was attained by the LPA electrons at a cumulative dose one order of magnitude lower than the dose obtained by pulsed X-ray irradiation. Interestingly, we observed areas where the LPA electron exposure had less damaging effect on melanocytes than on A375 melanoma cells.

Conclusions: Our data provide evidence of a differential response of tumor and normal cells exposed concurrently to electron FLASH irradiation. Current experiments are underway to characterize the comparative dose-dependent response of cancerous and tissue-specific normal cells to electron FLASH radiation. Future experiments are dedicated to the identification of key death pathways involved in cancer cells response to FLASH radiation and to the investigation of the molecular mechanisms related to the FLASH sparring effect.

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Advanced 3D Biomimetic Model Unveils Important Chemoresistance Traits in Melanoma

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Introduction: The clinical effectiveness of many anticancer drugs often proves inadequate, despite promising outcomes in preclinical studies. This is due to the limitations and lack of reliability of traditional 2D in vitro models used for drug testing. There is a constant need to develop advanced tools that accurately simulate the structural and functional complexity of a tumor, and enable a more thorough investigation of the cellular and molecular mechanisms behind the failure of current anticancer therapies. In this study, we aimed to develop an innovative in vitro 3D biomimetic platform, incorporating hydrogel-embedded multicellular spheroids, composed of murine B16.F10 melanoma cells, macrophages, fibroblasts and endothelial cells. This platform, advancing from TRL2 to TRL4, is designed to accurately replicate the complexity of the melanoma microenvironment and its chemoresistance, acting as a valuable tool for drug screening and the study of intratumor cellular interactions.

Materials and methods: The 3D biomimetic platform was created using the liquid overlay technique by seeding B16.F10 cells, 2H11 endothelial cells, primary fibroblasts, and macrophages in a 1:1:1:4 ratio within a 1% commercial extracellular matrix. These cells were selected for their key roles in melanoma progression and chemoresistance. We validated the platform using doxorubicin (DOX) at a sub-IC50 concentration, a drug to which melanoma patients are resistant. Cell viability within spheroids was assessed by measuring acid phosphatase activity. Chemoresistance was evaluated by assessing the expression levels of proteins involved in melanoma progression/apoptosis (via western blot), oxidative stress marker production (total antioxidant capacity-TAC/catalase-CAT activity via spectrophotometry, malondialdehyde-MDA levels via HPLC), pro-angiogenic/pro-inflammatory proteins (by RT-PCR and/or protein microarray) and the activity of matrix metalloproteinases (via zymography). Additionally, transcriptomic analysis was conducted to monitor changes in gene expression following DOX treatment.

Results: Our results revealed a complex response of the 3D biomimetic platform to DOX treatment, marked by reduction of the expression level of in HIF-1α, pAP-1 c-Jun, and pAkt proteins, alongside unmodified Bax and Bcl-2 expression levels, compared to their levels in Control (untreated platform). Despite unchanged TAC and MDA concentration, catalase activity increased, and MMP-9 activity was upregulated, while MMP-2 remained stable. Cytokine analysis showed selective increases upon DOX treatment in the expression level of G-CSF, GM-CSF, and Leptin, with unmodified levels of other key inflammatory and angiogenic factors supporting tumor cell progression and chemoresistance. Transcriptomic analysis revealed DOX-induced overexpression of genes linked to apoptosis, cell signaling, and matrix remodeling.

Conclusions: Overall, the developed 3D biomimetic platform, which includes various cell types from the tumor microenvironment (TME), exhibited traits of DOX-chemoresistance, underscoring its significant potential as a tool for drug screening and research on TME-associated chemoresistance.

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Rose gall induction by *Diplolepis* wasps: A hypothesis based on the possible involvement of phytoplasmas

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Introduction: Plant galls are abnormal somatic deformities induced by external biotic agents, such as viruses, bacteria, fungi, and insects, resulting in highly specialized structures that benefit the gall-inducing organisms. Unlike animal cancers, which are often driven by internal factors, plant galls are exclusively caused by external organisms, leading to diverse and complex tissue growths that can serve as diagnostic markers for identifying the inducing agents. The mechanisms behind gall formation, especially those involving insect-induced galls, remain largely unexplored. Our study investigates the potential role of phytoplasma within the microbiomes of gall-inducing wasps as a critical factor in the induction of galls on wild roses.

Materials and methods: By conducting a comprehensive analysis of the microbiomes of these insects, with a focus on identifying phytoplasma presence, we aim to elucidate the interactions between phytoplasmas, gall-inducing wasps, and host plants.

Results: We identified the presence of phytoplasmas in the microbiomes of *Diplolepis* species, specifically in *D. mayri*, *D. eglanteriae*, and *D. nervosa*. However, no evidence of phytoplasmas was detected in D. spinosissimae. Beyond, we have not detected phytoplasmas in the different host plant phytobiomes.

Conclusions: The lack of phytoplasma in *D. spinosissimae* and the phytobiomes may be contributed to the low resolution of the sequencing effort. Thus, further investigations are needed. Understanding these interactions could shed light on the evolutionary dynamics of plant-gall relationships and reveal molecular mechanisms underlying gall formation. The findings may also have broader implications for plant pathology, pharmacology and cosmetics.

Snapshot on the metabolism of Halomonas elongata DSM 2581T during the synthesis of polyhydroxybutyrate.

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Introduction: Polyhydroxybutyrate (PHB) is a naturally occurring polyester produced by various microorganisms as a response to nutrient limitation [1]. The biosynthesis of PHB involves some key enzymes from the butanoate metabolic pathway mainly acetyl-CoA C-acetyltransferase (PhaA), acetoacetyl-CoA reductase (PhaB), and poly[(R)-3-hydroxyalkanoate] polymerase (PhaC). While the molecular mechanisms underlying PHB production in halophilic organisms remain largely unexplored, this study aims to elucidate the metabolic changes occurring in *Halomonas elongata* during PHB synthesis under nutrient-limited and high-salinity conditions.

Materials and methods: *H. elongata* strain DSM 2581^T was cultured in both nutrient-rich and nutrient-limited culture media containing 8% w/v NaCl. The nutrient-limited media contained 1% w/v Glucose and 0.1% w/v yeast extract. Biomass samples were collected at 24, 96, and 120 hours for RNA extraction and sequencing. Total RNA was extracted using the RNeasy PowerSoil Total RNA kit provided by Qiagen. RNA sequencing was performed at Macrogen with the Illumina NovaSeq 6000. Raw sequence data were quality-controlled with FastQC v0.11.7 and trimmed with Trimmomatic 0.38. Subsequently, short RNA reads were aligned to the reference genome using Bowtie 1.1.2., and gene expression levels were quantified using HTSeq 0.10.0.

Results: The excess of carbon source in the medium resulted in a constant upregulation of sugar porter family MFS transporter-encoding gene in all the given experimental conditions compared to the control. Regarding the glycolysis only glyceraldehyde-3-phosphate dehydrogenase-encoding gene was upregulated, all the other genes encoding the glycolytic enzymes were variably regulated without a consistent pattern. Regarding the Krebs cycle, all the genes that encode the enzymes were downregulated. During the PHB synthesis only the *phaA* gene was downregulated and the *phaC* was upregulated.

Conclusions: This study reveals that PHB synthesis in *H. elongata* does not follow the typical pattern. Genes encoding for sugar transport proteins are upregulated across all three experimental conditions, indicating that sugar uptake is facilitated during the cultivation period. Surprisingly, the phaA, which encodes the first enzyme involved in PHB synthesis, is downregulated. This unexpected finding suggests that the metabolic process underlying PHB synthesis in *H. elongata* might be influenced by other factors (i.e. yeast extract). A detailed investigation is needed to elucidate the complete metabolic pathways involved in this process. Further experiments with a more refined experimental design may be crucial to better understand the primary effects of nutrients on cellular metabolism.

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New Polydimethylsiloxane (PDMS) modified interfaces: physical-chemical characteristics and *in vitro* effect on cell behaviour

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Introduction: One strategy to develop new and more efficient biodevices used as breast implants is based on surface physical-chemical modifications in order to enhance biocompatibility and prevent undesired biological responses [1, 2]. We propose a new scaffold based on PDMS with modified interfaces and loaded with active compounds for preventing inflammatory and fibrotic processes.

Materials and methods: The physical-chemical characteristics of the newly proposed PDMS functionalized scaffold obtained by Matrix-Assisted Laser Evaporation (MAPLE) method [3] were evaluated by SEM, Atomic Force Microscopy, Contact angle, Surface energy, Fourier Transform Infrared Spectroscopy and X-ray Photoelectron Spectroscopy. Biological in vitro studies such as cell adhesion, proliferation, morphology and immunological investigations of cells grown on PDMS surfaces were performed on human macrophages and fibroblasts, cells involved in inflammatory and fibrotic processes. The inhibition of bacterial growth and biofilm formation was assessed on relevant gram-negative bacteria strains.

Results: The results obtained after evaluation of the physical–chemical properties of the new coatings revealed that the MAPLE technique proposed has the advantage of achieving homogeneous, stable and moderate hydrophilic thin layers onto hydrophobic PDMS. Moreover, this approach does not require any pre-treatment, therefore avoiding the major disadvantage of hydrophobicity recovery. Biological investigation evidenced the reduction of the adhesion and viability of human macrophages and fibroblasts by ~40% and ~50%, respectively on the modified surfaces of PDMS loaded with active compounds as compared to unmodified scaffold. A similar cellular behavior on the modified surfaces was observed in the presence of either inflammatory or profibrotic stimuli. Moreover, a decrease of proinflammatory cytokines (IL-6 and TNF-alpha) was observed in *in vitro* models of inflammation. In the presence of TGF-beta, PYR reduced collagen synthesis and fibronectin deposition. In the case of a fibrotic process induced by TGF-beta, the expression of collagen marker was reduced by the presence of scaffold loaded with biological active compound. A reduction of bacterial growth associated with inhibition of biofilm formation was obtained on PDMS modified surfaces by using *E. coli* gram negative bacterial strain and confirmed by SEM analysis.

Conclusions: All these highlighted the potential for the new PDMS interfaces obtained by MAPLE to be used in the biomedical field to design PDMS-based implants exhibiting long-term hydrophilic profile stability and better mitigating foreign body response.

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Targeting Hypoxia-Inducible Factor-1 to Suppress Angiogenesis and Apoptosis in B16.F10 Murine Melanoma Cells *in vitro*

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Introduction: Conventional therapies and even recently discovered targeted drugs lack the capability to overcome the challenges related to the rapid development of resistance to treatment of metastatic melanoma. Resistance is mainly acquired through the activation of hypoxia-inducible factor-1 (HIF-1) under low oxygen conditions found within the tumor microenvironment. Moreover, B16.F10 murine melanoma cells have constitutive expression of HIF-1 gene even under normoxic conditions, and have been associated with decreased sensitivity to chemotherapy drug doxorubicin [1, 2].

Materials and methods: Addressing this situation, we used an *in vitro* approach to develop an effective therapy for sensitizing melanoma cells to doxorubicin by small interfering RNA (siRNA)-mediated HIF-1α gene silencing. Melanoma cells were reverse transfected with an anti-HIF-1α siRNA and incubated for 24 hours under 1% O_2 conditions then treated with doxorubicin the following day and incubated for additional 24 hours.

Results: Our preliminary data have shown that HIF-1α gene knockdown improved doxorubicin efficacy by inhibiting angiogenesis and inducing apoptosis.

Conclusions: Collectively, our results suggested that inhibition of HIF-1α holds promise as an important therapeutic approach to overcome B16.F10 murine melanoma cell resistance to doxorubicin.

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3D tissue-like scaffolds fabricated by two-photon polymerization for melanoma cell invasion evaluation

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Introduction: Three dimensionally (3D) engineered scaffolds could represent a viable alternative to investigate cancer cells behavior in physiologically-relevant configurations. It is known that cancer cell passage through confined spaces leads to cell-specific morphological deformations. The ability of cells to deform depend on their plasticity and actin remodeling capacity, which support their migration and metastasis. Herein, we fabricated polymeric tissue-like scaffolds consisting of narrow confined micropores and tested the invasive potential of melanoma cancer cells on collagen and non-coated substrates.

Materials and methods: The scaffolds were fabricated in SU-8 photoresin *via* two-photon polymerization (2PP), a maskless laser writing method, using a Nanoscribe Photonic Professional platform. We proposed 50×50×50 μm^3 scaffolds with three different pore dimensions to act as confined spaces for cancer cell invasion studies. A375 melanoma cells were used to quantitatively evaluate the cellular adhesion and invasion at the interface with the scaffolds, coated or not with collagen by analyzing cell density, cytoskeleton pattern and focal adhesions (FAs) maturation. We further evaluated cell trajectories in unrestricted environment using live cell imaging with the aim to test the correlation of cancer cell invasive potential in confined spaces with cell motility.

Results: Scanning electron and fluorescence microscopy images revealed that melanoma cells exhibited an increased adhesion when collagen coating was applied (Fig. 1). A two times higher cellular affinity around collagen-coated scaffolds was observed in comparison with non-coated scaffolds. An increased cell body penetrating area was evidenced as the pore size increases from 0.70 to 1.66 µm, both on collagen coated and non-coated samples. The quantification of vinculin-positive FAs developed either inside the scaffold or at the border showed a higher number of mature FAs on non-coated samples. The time lapse microscopy evidenced a higher migration velocity on collagen-coated substrates that correlates with the cell invasiveness within collagen-coated scaffolds.

Conclusions: 3D polymeric scaffolds were fabricated by 2PP and coated with collagen to better mimic a bioenvironment suitable for testing the invasive potential of melanoma cancer cells in constrictive spaces. We noticed two times higher cellular affinity around collagen-coated scaffolds based on the number of cell nuclei and number of focal adhesion points anchoring around the borders or inside the scaffolds. Our studies have evidenced a higher cellular motility on collagen-coated substrates that correlates with cell invasion within collagen-coated scaffolds.

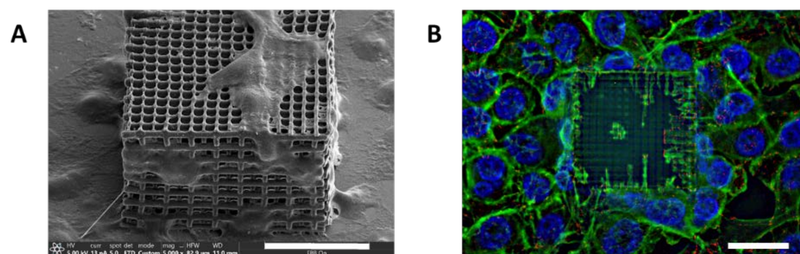

Figure 1 (A) SEM image of SU-8 collagen-coated scaffold surrounded by melanoma cells and **(B)** the corresponding fluorescent microscopy top-view (actin cytoskeleton – green, nuclei – blue, focal adhesions - red). Scale bars are of 25 µm

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Using subtherapeutic doses of doxorubicin to assess development of adaptive and resistance mechanisms in a murine melanoma model

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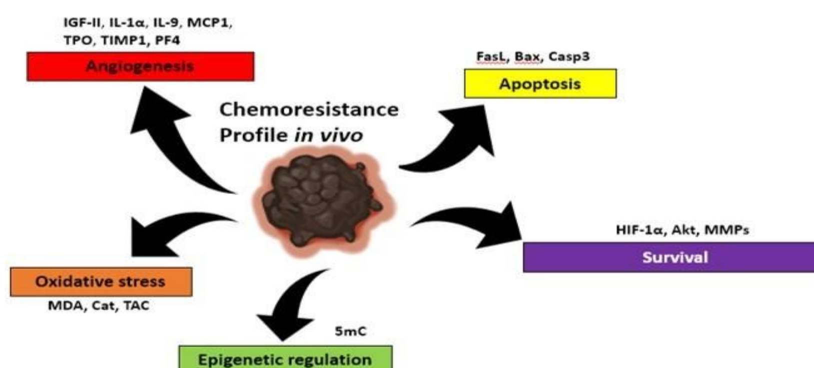
Introduction: Resistance to treatment poses a great challenge to melanoma patient recovery, by being either intrinsic or acquired, and thus making long-term control of the disease a very difficult task. Previous studies have shown that exposing cancer cells to lower doses of doxorubicin over time can lead to the development of drug resistance via various mechanisms, including changes in drug efflux, alterations in drug targets, and activation of survival pathways. Our aim was to explore the role of melanoma microenvironment in the development of adaptive and resistance mechanisms leading to the well-known doxorubicin-related chemoresistance profile in melanoma. Thus, we assessed *in vivo* the effect of subtherapeutic doses of doxorubicin regarding major chemoresistance hallmarks: angiogenesis, apoptosis, oxidative stress, epigenetic regulation, and survival [1, 2].

Materials and methods: C57BL/6 mice 6 to 8-week-old were inoculated with B16.F10 cells *s.c.* in the right flank. Treatments were administered at day 8, 10 and 12 after cell inoculation and consisted of 0.5 mg DOX/kg either free (DOX), to obtain and validate the chemoresistance profile, or incorporated in B16.F10 derived extracellular vesicles (PEG-EV-DOX) to restore chemosensitivity based on the superior targeting capacity. In tumor lysates we explored the molecular mechanisms underlying DOX chemoresistance using various techniques, including qPCR, protein array, Western blotting, HPLC, spectrophotometry, and ELISA.

Results: Our primary findings revealed that DOX chemoresistance was linked to a pro-angiogenic profile, marked by the substantial upregulation of IGF-II, IL-1α, IL-9, MCP1, TPO, TIMP1, and PF4 proteins. Although subtherapeutic DOX treatment induced an upregulation of the pro-apoptotic factor FasL, the trend of downregulation of major proapoptotic players Bax and Casp3 indicated resistance to apoptosis. Conversely, PEG-EV-DOX treatment reinstated the pro-apoptotic status by upregulating Bax and Casp3. While subtherapeutic dose of free DOX did not significantly impact the redox status, DOX treatment resulted in elevated gene expression of tumor cell survival factors HIF-1α and Akt, along with an increase in the invasion marker MMP-2 on both gene and protein levels. Regarding epigenetic modifications, global methylation was higher in the DOX chemoresistant group compared to the Control and PEG-EVDOX-treated groups.

Conclusion: The development of adaptive and resistance mechanisms to doxorubicin in melanoma is strongly modulated by the tumor microenvironment, which contributes decisively to chemoresistance by pro-angiogenic and anti-apoptotic features, modulation of redox status, upregulation of global DNA methylation and survival-related transcription factors.



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Anthocyanins – from chemistry to biological effects

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Introduction: Anthocyanins are a class of water-soluble plant pigments that impart a wide range of colors, from pink, red, purple or even black, to flowers, fruits and vegetables, being also associated with several health promoting effects. However, their low stability storage and processing, as well as the reduced bioavailability represent challenges for their use in the food and pharmaceutical industry [1]. These issues were addressed in the current study using anthocyanin rich extracts, in order to better understand the impact of various factors (pH, temperature, light) on their stability. The bioaccessibility, antioxidant, enzyme inhibitory and the *in vitro* protective effects of anthocyanins-rich extracts from berries were further investigated.

Materials and methods: *Amelanchier lamarckii* berries cultivated in Romania were used to obtain anthocyanins-rich extracts (AE). Characterization of the extracts and the stability studies were performed using LC-ESI+-MS and HPLC-PDA. Antioxidant activity was determined by ABTS, FRAP and CUPRAC assays while the bioaccessibility was evaluated by INFOGEST protocol. The inhibition of tyrosinase, α-Glucosidase and cholinesterase by AE was determined compared to known inhibitors and the protective effect against hyperglycemic stress in retinal pigment epithelial cells was also assessed.

Results: *Amelanchier lamarckii* extract represents an interesting source of bioactive phenolic compounds, among them cyanidin-based anthocyanins being major constituents. AE exhibited good stability (over 78% retention) at all pHs (1.1, 5.1, 8.0) and low temperatures (<37 °C), but it decreased significantly starting from 60°C, regardless of the pH. At lower temperatures and in the absence of light, the glycosylated derivatives were more stable toward the variation of pH compared to Cy, especially for longer exposure times. At high temperatures (>37°C), however, Cy had better retention than the glycosylated form, probably due to the thermally induced hydrolysis of the glycosylated derivatives. A thermal treatment at 60°C or boiling (100°C) for 1 hour in mild acidic pH (5.1) resulted in a loss of about 23% and, respectively, 46% of total anthocyanins from juneberry extract, while for longer times (8-24 hours), anthocyanins were completely degraded. The extract showed antioxidant capacity 323.99 µmol Trolox/g FW (CUPRAC assay) and 4.10 µmol Fe^{2+}/g FW (FRAP assay) and low to moderate inhibitory activity, tyrosinase (IC50 = 8.843 mg/mL), α-glucosidase (IC50 = 14.03 mg/mL), and acetylcholinesterase (IC50 = 49.55 mg/mL). The total concentration of polyphenols was six fold and ten folds lower after the gastric phase, and respectively the intestinal phase of the *in vitro* digestion, with anthocyanins displaying the lowest bioaccessibility. In a simulated diabetic retinopathy model, AE exerted a protective effect in high-glucose conditions (30 mM), but in extreme glucose conditions (60 mM), the protective effect of AE on the viability of RPE cells was reduced.

Conclusions: Due to their antioxidant and protective effects, AE from *A. lamarckii* could be used as food colorants or nutraceuticals, but the processing and storage conditions should be carefully controlled in order to minimize the degradation of the bioactive compounds. Development of delivery systems using biodegradable and biocompatible materials could be a strategy to improve their stability and bioavailability.

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Viscum album L. phytochemical profile, antioxidant capacity and in vitro wound healing potential

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Introduction: Numerous chemical and pharmacological research has shown that Viscum extracts and their various forms contain a wide variety of compounds, such as lignans, amino acids, lectins, viscotoxins, amines, alkaloids, polyphenols, flavonoids, and polysaccharides [1]. The bioactive components of mistletoe, flavonoids and phenolic acids, have been used for disease prevention, especially in relation to oxidative stress-related conditions like cancer [2]. The primary objective of our research was to identify the polyphenolic compounds and explore the potential of *Viscum album* L. extract for wound healing by an *in vitro* experiment. An additional objective was to evaluate the mistletoe extract's antioxidant potential using classical spectrophotometric techniques, and a novel approach based on the environmentally friendly synthesis of nanoselenium particles (NSePs).

Materials and methods: The polyphenols profile of mistletoe was identify by High performance chromatography method coupled with mass spectroscopy (HPLC-DAD-MS-ESI$^+$) and total phenols and flavonoids content was determined by spectrophotometric assay. The antioxidant capacity of extract was evaluated by three different methods (DPPH, FRAP, and TEAC assay). In addition, the potential of mistletoe extract to reduce sodium selenite (Se^{4+}) to NSePs was highlighted. The wound healing potential of the *V. album* L. extract was evidence by using the "scratch" technique by an *in vitro* experiment employing normal human dermal fibroblasts (NHDF).

Results: The 16 bioactive compounds from polyphenols class that were separated and tentatively assigned by HPLC, based on retention times, mass/charge ratio (m/z) and their main fragments. It was noticed that the mistletoe extract is richer in dihydroxybenzoic acid having a concentration of 2.864 ± 0.03 mg/g dw, than in hydroxicinnamic acids. The predominant flavonol found in mistletoe leaves was Isorhamnetin-glucuronide. It was demonstrated that mistletoe extract has antioxidant properties among the three antioxidant techniques used. The reducing power of the mistletoe was highlighted by obtaining red NPSe. The apparent zeta potential of the SeNPs was recorded at a maximum value of -24.5 mV, indicating that they do not form aggregates in solution that lead to a stable dispersion. The greatest absorbance of the SeNPs was seen at 270 nm. The diameter of SeNPs was approximately 159 nm, and TEM confirmed the spherical shape of the particles. A statistically significant ($p < 0.05$) increase in the percentage of wound closure was observed at a dose of 100 μg/mL mistletoe extract from 24-36 hours as opposed to 12–24 hours.

Conclusions: Aqueous extract of mistletoe, which is rich in antioxidant compounds, can heal an artificially created lesion in vitro and is not harmful to NHDF at the doses evaluated.

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POSTER

PRESENTATIONS

Bioinformatic analysis of the genes correlated with prostate adenocarcinoma differentiation

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Introduction: Gleason Score (GS) is widely used in the management of prostate cancer to assess prognosis and determine treatment strategy [1]. This study aimed to correlate gene expression with the differentiation of prostate adenocarcinomas, as indicated by the GS.

Materials and methods: In this study, whole transcriptome data from 497 prostate cancer patients included in The Cancer Genome Atlas (TCGA) was analyzed. Gene ontology and STRING network analyses were conducted to identify molecular functions and pathways associated with GS and immune cell enrichment was assessed using CIBERSORTx. A combinatorial approach employing the k-Nearest Neighbors (kNN) classification algorithm was used to assess gene combinations predictive of GS and produced an eight-gene signature, which was validated using the Human Protein Atlas (HPA).

Results: Higher Gleason scores correlated with increased age and advanced pathological stages. Gene ontology analysis showed that higher GSs were associated with DNA damage response, telomere lengthening, and cell division. Immune cell analysis revealed higher counts of M0 macrophages in tumor tissues, regardless of differentiation, and GS-dependent increases in M2 macrophages. STRING analysis identified central nodes like CDC20 and PLK1 positively correlated with GS and *CAV1*, *CALM1*, and *PAK3* negatively correlated with GS. The eight-gene signature composed of *FOXS1*, *NSD2*, *CDC42EP4*, *AGL*, *VPS36*, *TMLHE*, *ANGPT1*, and *C22orf23* showed high accuracy in differentiating normal, low-Gleason, and high-Gleason tissues.

Conclusions: This study highlights significant molecular pathways and immune cell changes linked to high Gleason grades in prostate cancer. The eight-gene expression signature proposed offers a promising tool for improving prostate cancer classification and prognosis assessment.

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Purification of Fc-fusion proteins without: Chromatography, polymers, membranes or specific-ligands

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We introduce a new concept and a potentially general platform for purification of Fc-fusion proteins that does not rely on any resins, chromatographic media, membranes or specific-ligands, rather, makes use of aromatic [metal:chelator] complexes. Our technology captures the target proteins quantitatively *via* [cation:pi] and [pi:pi] interactions and allows their recovery at high yields (>80%, by densitometry) and purity (≥90%, by SDS-PAGE), while preserving their secondary structure (by circular dichroism, CD), enzymatic activity and monomeric state (by dynamic ligand scattering, DLS). The entire process is performed at pH 7 thereby avoiding complications that derive from exposure to harsh acidic conditions (*e.g.*, aggregation, partial denaturation). The cost-effectiveness and simple integration into future, industrial-scale downstream processing of therapeutic-grade biopharmaceuticals, will be discussed. In future, we are planning to apply the same purification technology on different type of therapeutic proteins as an efficient purification plat form while applying the necessary modification.

Simvastatin Effects on Lipid Metabolism of Normoxic B16.F10 Murine Melanoma Cells

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Introduction: As a competitive inhibitor of the rate-limiting enzyme of cholesterol biosynthesis, 3hydroxy-3-methylglutaryl-Coenzyme A reductase, (HMG-CR), simvastatin (SIM) has demonstrated antitumor effects at high doses [1-3]. Moreover, our previous study has proved that under normoxic conditions, the expression of hypoxia inducible factor 1 (HIF-1) - a central regulator of cancer cell metabolism, was inhibited by SIM [2-3].

Materials and methods: As a follow-up of our earlier findings, in this study we explored the impact of SIM at IC50 on the lipid metabolism of B16.F10 murine melanoma cells, under normoxic conditions. In close connection with SIM's primary pharmacological role, we explored the lipophilic statin effect on the cholesterol biosynthesis as well as fatty acid metabolism. Thus, RNA-seq analysis for mRNA expression was performed, while western blot analysis was used to assess the levels of the key proteins such as, HIF-1α as metabolic regulator as well as proteins involved in lipid metabolism pathways (fatty acid synthase (FASN), stearyl-CoA desaturase 1 (SCD1), and acyl-Coenzyme A oxidase (ACOX3)).

Results: Our data suggested that SIM significantly modulated the expression of genes encoding for multiple proteins involved in lipid metabolism.

Conclusions: These data suggest that SIM caused a transition toward unsaturated fatty acid metabolism and isoprenoid production favoring melanoma cell proliferation and metastasis.

Acknowledgements: This work was funded from L'Oréal - UNESCO "For Women in Science" Fellowship Programme (no. 914/26.11.2020), and UEFISCDI grant PN-III-P2-2_1-PED-2021-0411 (No. 659PED/2022) granted to dr. Alina Sesarman.

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Deciphering the cellular effects of a non-visual opsin receptor, OPN3

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Introduction: Opsin 3 (OPN3) is a member of the light-sensitive, retinal-dependent opsin family, which is widely expressed in human tissues, including the brain, testis, liver, placenta, heart, lung, kidney, pancreas, skeletal muscle and skin. Recent studies have elucidated some of the main functions of OPN3, including its role in light-mediated glucose uptake, mitochondrial respiration and fatty acid metabolism in brown adipocytes[(1)]; its regulation of melanogenesis in melanocytes by the inhibition of MC1R-mediated cAMP signalling[(2)]; its involvement in the development of insulin resistance and obesity; and its status as a poor prognostic factor in various types of cancer if overexpressed[(3)]. Given its extensive metabolic functions and orphan GPCR status, OPN3 is currently one of the most studied opsins, as the identification of agonists/antagonists may be important for novel drug development for obesity and type-2 diabetes. The aim of our study was to characterize the cellular effects of heterologously expressed OPN3 receptor, as a required step in the identification of compounds with modulatory effects on OPN3 activity (agonists and antagonists).

Materials and methods: The expression and cellular distribution of the receptor were examined using western blotting and semi-quantitative immunocytochemistry. The functionality of the receptor was evaluated by monitoring the inhibition of signaling pathways, specifically adenylyl-cyclase/cyclic adenosine monophosphate (AC/cAMP) and serum response element/factor (SRE/SRF), as indicated by bioluminescence signals recorded in HEK293T cells that expressed the receptor heterologously.

Results: Human OPN3 expression in HEK293T cells was verified through Western blotting, while its cellular localization was demonstrated using immunocytochemistry. The data indicate that OPN3 is expressed at the plasma membrane in a glycosylated form, thereby confirming that HEK293T cells provide a suitable model system for further investigation. At the functional level, our results demonstrate that expression of OPN3 results in an inhibition of several cellular signaling pathways such as AC/cAMP pathway in a G-protein independent manner as demonstrated by the lack of sensitivity to specific G-proteins inhibitors such as pertussis toxin (PTX) and OZITX (a broader inhibitor of G-proteins). Building on this observation, we hypothesized that cotransfection of the receptor with GRK proteins should reverse its inherent inhibitory effect, based on the premise that serine/threonine kinases phosphorylate the activated GPCR, desensitizing it. Nevertheless, contrary to this hypothesis, GRK2 and GRK4, the kinases we tested, did not reverse the inhibitory effect of OPN3, which appeared to be independent of the degree of receptor phosphorylation. Consistent with the observed inhibition of AC/cAMP pathway, the expression of OPN3 resulted in a drastic inhibition of SRF activity, a phenomenon also insensitive to G-proteins inhibitors.

Conclusions: Our preliminary results demonstrate that the heterologous expression of human OPN3 in HEK293T cells is associated with profound changes of specific signaling pathways, leading to the inhibition of AC/cAMP/SRE and SRF-activities in a ligand- and G-protein independent manner. These results are important for future compound screening campaigns aimed to discover modulators of OPN3 activity. It is therefore a matter of considerable scientific interest and importance to investigate the cellular and functional roles of specific non-visual opsins, such as OPN3, which may interact with light either directly or indirectly. Such research may yield significant clinical and socioeconomic value.

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Analysis of the hsa-miR-130a and hsa-miR-365a -3p/-5p ratios in prostate cancer biofluids

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Introduction: Prostate cancer (PCa) ranks as the second most common and fifth most lethal cancer in men. Screening and diagnosing PCa involves measuring blood prostate-specific antigen (PSA) levels, a test with high sensitivity but unsatisfactory specificity. As PSA's high rate of false positives leads to overdiagnosis and unnecessary biopsies, there is a stringent need for alternative, non-invasive, more specific, and sensitive biomarkers for PCa diagnosis. Circulating microRNAs, particularly the guide strands, have emerged as promising stable and non-invasive molecular markers for PCa. In addition, due to arm switching, the passenger strands may also become functional and associated with the disease [1-3].

Materials and methods: In this study, we measured the plasma and urine expression of the 3′ and 5′ strands of two microRNAs, hsa-miR-130a and hsa-miR-365a, known for their guide strands differential expression in PCa. We used the Qiagen miRNeasy Serum/Plasma kit to isolate microRNA-enriched total RNA and individual TaqMan assays to perform RT-qPCR. We quantified the absolute copy number of the guide and passenger strand microRNAs in plasma and urine samples from PCa patients and controls.

Results: When comparing patients to controls, we observed consistent strand expression dysregulations and changes in 3p/5p ratios across the two biological samples. Correlation analyses revealed paired associations between strand expression level, 3p/5p ratios, and clinical characteristics across the two types of biospecimens.

Conclusions: Our findings point towards microRNA-specific mechanisms altering the 3p/5p kinetics ratio in the plasma and urine of PCa patients.

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GPR27 modulates Hepatitis B Virus internalization

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Introduction: Hepatitis B Virus (HBV) causes 800,000 deaths annually among 296 million chronically infected individuals worldwide, and no curative treatment is currently available. Previous research suggests a tight interdependence between the HBV life-cycle and host cell metabolism. Unraveling the molecular details of these interactions could lead to the discovery of new antiviral targets and therapeutic strategies. G protein-coupled receptors (GPCRs) form the largest superfamily of human membrane receptors, playing a crucial role in signal transduction via the activation of intracellular G proteins. GPCRs regulate various physiological processes and are among the most significant pharmacological targets. We recently discovered that GPR27, a member of this family, is expressed in hepatic cells. Our hypothesis is that GPR27 may influence HBV infection by modulating host cell pathways involved in the viral life-cycle.

Materials and methods: Transient down-regulation of GPR27 were conducted in hepatoma cell lines. GPR27 silencing in HepG2-NTCP cells, permissive for HBV infection was achieved using 50 nM siRNA for 48 hours and validated with q-RT-PCR. **HBV infection**. HepG2-NTCP cells were incubated with HBV at a multiplicity of infection (MOI) of 300 genome equivalents/cell, following GPR27 silencing and harvested after 10 days. **HBV infection** was quantified by measuring the amounts of secreted HBV antigens. The Monolisa HBsAg Ultra Kit was used to measure the amount of HBV subviral particles (SVPs) released, according to the manufacturer's protocol. HBeAg secretion was monitored using the Monolisa HBe Ag-Ab PLUS kit. **Viral DNA extraction** was performed using the Phenol/Chloroform/Isoamyl Alcohol Extraction protocol, and qPCR was performed with HBV-specific primers to determine the amount of HBV nucleocapsids.

Results: Our results suggest that GPR27 downreglation could inhibit HBV entry, leading to a decreased infection rate, while no significant effect on late life-cycle steps was observed.

Conclusions: In this study, we have established that GPR27 is a novel host factor involved in HBV infection. Future research will focus on elucidating the molecular mechanism of this inhibition and identifying GPR27-targeting drugs with antiviral properties.

GPR75 receptor trafficking and its role in insulin secretion

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Introduction: G Protein-Coupled Receptor 75 (GPR75) is a receptor from the GPCR family, which is the largest family of membrane proteins and a rich source of targets for the pharmaceutical industry [1]. GPR75 might be involved in insulin secretion, potentiating secretion and preventing glucotoxicity-induced beta cell dysfunction [2]. Recently it was shown that certain truncated variants of GPR75 (especially the truncated form at Gln234* of GPR75 = GPR75 MUT) were associated with 1.8 kg/m^2 lower body mass index (BMI), 5.3 kg lower bodyweight, and 54% lower odds of obesity in heterozygous carriers, results confirmed in mice with diet-induced obesity [3]. To further understand the GPR75 biological function, we set out to study the processes of synthesis, trafficking and degradation of GPR75 WT and GPR75 MUT and to investigate its role in insulin secretion.

Materials and methods: The experiments were carried out in the INS-1E cell line, a model for insulin secretion in diabetes studies. Firstly, the cellular localization of GPR75 WT and GPR75 MUT was determined by an immunofluorescence experiment. Also for this purpose, a cellular subfractionation was performed on the Nycodenz gradient. In order to determine whether GPR75 WT and GPR75 MUT are glycosylated digestion with Endo H and PNGase F was performed. Then, the half-life of both forms of the receptor was established by a cycloheximide chase assay. Furthermore, the degradation pathways of GPR75 WT and GPR75 MUT were investigated by treatment with lysosomal/proteasomal inhibitors. Finally, the role of GPR75 WT and GPR75 MUT in insulin secretion was explored through ELISA and Western Blotting techniques.

Results: The fluorescence microscope images revealed that GPR75 WT and GPR75 MUT are predominantly present at the plasma membrane, but also in intracellular compartments, GPR75 MUT appearing to be retained at the ER level. Treatment with EndoH and PNGase F of INS-1E cells showed that most of the GPR75 WT is glycosylated in the Golgi with less than 20% retained in the ER, while GPR75 MUT N-glycans remain unprocessed. Treatment with cycloheximide led to the determination of the half-life of the GPR75 WT, which is about 2 hours, while, surprisingly, GPR75 MUT is much more stable over time. Moreover, it has been confirmed that GPR75 WT and GPR75 MUT are degraded by the lysosomal pathway, but also by the proteasomal pathway. Fractionation experiments showed that both forms of the receptor are present in mature insulin granules. Regarding the role of GPR75 in insulin secretion, the results of the glucose stimulation experiments showed that overexpression of GPR75 WT increases glucose-stimulated insulin secretion, while GPR75 MUT shows an opposite effect.

Conclusions: This research investigated the processes of synthesis, trafficking and degradation of the GPR75 receptor and its role in insulin secretion. Further studies are needed to better understand the trafficking of the GPR75 and if this receptor could become a new therapeutic target in the treatment of type 2 diabetes.

Inhibiting TGF-β signaling restores the function of cytokine-activated Natural Killer cells

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Introduction: Natural Killer (NK) cells can be activated ex vivo with cytokines in order to become more efficient at killing cancer cells prior to adoptive transfer into cancer patients. Despite the strong potential of NK cells to kill tumour cells, they infiltrate very little in solid tumours, the tumour microenvironment contributes through a number of mechanisms to the suppression of NK cell activity, one of the most important mechanisms being the excessive production of inhibitory cytokines, such as transforming growth factor beta (TGF-β). This immunosuppressive microenvironment ultimately limits the antitumor efficacy of NK cell therapy. In this study, we assessed the use of a TGF-β receptor type I/II inhibitor, LY2109761, in preserving the function of cytokine-activated NK cells exposed to pathologic levels of TGFβ in vitro models of cancer. Using melanoma and lymphoma cell lines, we show that the TGF-β driven impairment of NK cell functionality is mitigated by LY2109761.

Materials and methods: We demonstrate this effect using functional assays by showing a preserved activated phenotype with CD69 and CD25 expression and enhanced production of interferon γ (IFNγ), granzyme B and perforin. Spleens were harvested from C57BL/6J 8-10 weeks old mice, male and female. From single-cell suspensions of spleens we isolated highly pure unlabeled NK cells by depletion of nontarget cells (Miltenyi Biotech). Freshly-isolated NK cells ($1x10^6$ cells/mL) were incubated overnight alone or in co-culture with B16F10 melanoma cells or lymphoma YAC-1 cells ($0.2x10^6$ cells/mL) in media supplemented with interleukin 12 (IL-12; 10ng/mL), IL-15 (10ng/mL), IL-18 (50ng/mL), 21 (50ng/mL), TGFβ (5ng/mL) and LY2109761 (10μM/mL). After incubation period the NK cells were washed and immediately incubated with fluorochrome-conjugated mAbs and used for assessing the flow cytometry analyses of NK cells. Stained cells were analysed with a FACSCanto II flow cytometer using DIVA software.

Results: Combination of IL-12, 15 and 18 induced higher expression of CD69 and CD25 activation markers and IFNγ production compared with the combination of IL-12, 15 and 21, while the highest production of granzyme B and perforin resulted after stimulation with IL-12/15/21. Exposure to TGF-β impaired cytokine-activated NK cell function, all the parameters showed decreased values. Despite exposure to TGF-β the cytokine-activated NK cell phenotype was preserved by the addition of LY2109761. Also, in coculture with tumour cells, we observed the same restoration of cytokine-activated NK cell function when LY2109761 was added.

Conclusions: Addition a small molecule inhibitor of TGF-β receptor type I/II restored the cytokineactivated NK cell function *in vitro*. Adoptive transfer of cytokine-activated NK cells in combination with TGF-β receptor inhibitor, LY2109761, can markedly improve their antitumor efficacy.

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A preliminary *in vivo* investigation of the effects of a combined immunotherapy on B16.F10 murine melanoma model

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Introduction: Immune checkpoint blockades (ICBs) have emerged as a key treatment for various types of cancers, showing encouraging results in difficult cases. ICBs target proteins involved in the crosstalk between cancer cells and immune cells, therefore blocking the cell death signaling inflicted upon immune cells. Melanoma is one of the best-suited candidates for ICB due to its high immunogenicity. However, tumor heterogeneity continues to limit success rates, indicating a need for therapy optimization. The aim of this study was to evaluate the efficacy of combined immunotherapy based on anti-PD-L1 antibodies and curcumin-loaded extracellular vesicles derived from stressed, peptide-pulsed dendritic cells in treating murine melanoma *in vivo*. This strategy exploits the efficacy of anti-PD-L1 therapies, the targeted delivery capability of extracellular vesicles, and the immunomodulatory properties of curcumin.

Materials and methods: A pilot *in vivo* study was conducted on C57BL/6 mice bearing B16.F10 melanoma tumors to assess the effects of anti-PD-L1 antibodies combined with EVs with curcumin (PEG-EV-CURC). The tumor volume and mouse body weight were monitored throughout the study. Post-treatment, tumors were excised, measured, and subjected to molecular analyses including the assessment of the level of expression of proteins involved in inflammation and apoptosis by western blot, of cytokines and growth factors involved in inflammation and angiogenesis by protein microarray, the activity of MMPs involved in invasion and metastasis by gelatin zymography, and the percentage of 5-methylcytosine, as a measured of epigenetic changes associated with tumor progression, determined by ELISA.

Results: The combined treatment led to a >50% inhibition of tumor growth compared to the growth of untreated tumors ($p < 0.0001$). Molecularly, the level of expression of critical proteins such as pAkt was significantly reduced (>50%), while the expression level of pNF-κB and iNOS, remained unchanged compared to control tumors. The combined therapy group exhibited a broad reduction in the expression of cytokines involved in inflammation and angiogenesis, including G-CSF ($p < 0.0001$), M-CSF ($p < 0.0001$), Eotaxin 1 ($p < 0.001$) and VEGF-A ($p < 0.01$), compared to the control group, with even more pronounced impact when compared to the group treated with anti-PD-L1 antibodies alone. As for the metastatic capacity, the MMP-2 expression levels were slightly elevated in the combined therapy cohort ($p<0.01$) compared to the control, while MMP-9 levels were not modified across all treatment groups. Furthermore, the combined therapy decreased cytosine methylation in tumors ($p < 0.01$) compared to the control group.

Conclusions: This combined immunotherapy shows potential in treating murine melanoma and may possibly be further optimized by adjusting the timing and dosage of administration of the encapsulated curcumin.

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Curcumin reverses irinotecan resistance in colorectal cancer cells: molecular insights from whole transcriptomic data

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Introduction: A significant challenge in the current colorectal cancer (CRC) treatment is the cancer's acquired resistance to conventional chemotherapy, such as irinotecan-based therapeutic grids. Bioactive constituents found in plants are a feasible solution for overcoming acquired resistance since specific compounds can resensitize chemoresistant cancer cells to certain drugs. One such compound is curcumin. Therefore, this study aims to evaluate the potential of curcumin to reverse acquired resistance to irinotecan in CRC cells and to identify the molecular mechanisms through which curcumin operates in this context of cancer resistance.

Materials and methods: An irinotecan-resistant cell line (DLD1_IRI-R) was obtained through successive exposures of DLD-1 cells to increasing concentrations of irinotecan, until cells would become resistant to to 20 µM irinotecan. To test the sensitization capacity of curcumin, a combined treatment of irinotecan in successive concentrations and curcumin at the constant low concentration of IC10 was employed. RNA extraction, cDNA synthesis and microarray analysis were employed to determine the whole transcriptome expression profiles of the parental DLD-1 cell line, the resistant variant, and the resistant cell line treated with either curcumin or irinotecan. The functional analysis of the gene expression patterns was performed using Ingenuity Pathway Analysis (IPA) software. Finally, the expression levels of the selected genes of interest were validated by RT-qPCR.

Results: DLD1_IRI-R cells were 1.78X more resistant to irinotecan than the parental cells (IC_{50} – DLD-1=212,5 µM; IC_{50} – DLD-1_IRI-R=378,8 µM). The sensitivity to curcumin remained the same after cells acquired resistance to irinotecan. The combined treatment of irinotecan in successive concentrations and curcumin at the constant low concentration of IC_{10} (11,55 µM) led to a decrease in the IC_{50} of irinotecan (118,3 µM), the resistant cell line becoming 2,95X more sensitive to the compound. The functional analysis of the expression profiles revealed that acquisition of irinotecan resistance in DLD-1 resistant CRC cell line is primarily determined by suppression of proliferation, driven by *EIF2AK3* overexpression, along with increased expression of drug efflux and drug metabolism genes, including *ABCC1*, *ABCG1*, and *AKR1B10*. Curcumin administration showed antiproliferative and pro-apoptotic effects on the DLD-1 irinotecan resistant cells, mainly through growth receptor and NF-kB pathway inhibition. Additionally, curcumin demonstrated the potential to reverse irinotecan resistance in the DLD-1 resistant CRC cell line by decreasing the expression of several genes involved in drug efflux and metabolism: *ABCB1*, *ABCG1*, *ABCC1*, *ABCC3*, and *AKR1B10*.

Conclusions: Curcumin has both antiproliferative and pro-apoptotic effects on irinotecan resistant cells and the potential to reverse irinotecan resistance by downregulating genes implicated in drug efflux and drug metabolism.

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Unraveling the Complexities of Cerebral Cortex Peroxidase Behavior on Parkinson's Disease: Examining Correlations with Inflammation and Redox Homeostasis

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Introduction: The mechanisms underlying neuronal degeneration in Parkinson's disease (PD) are complex and not yet fully understood. Current research indicates that oxidative damage and mitochondrial dysfunction play significant roles in the degeneration of dopaminergic neurons within the striatum [1]. A previous study revealed an increase in the activity of glucose-6-phosphate dehydrogenase (G6PD) and a 2.0-fold rise in glutathione (GSH) levels in the brains of individuals with PD [2]. The present challenge lies in deciphering the relationship between cell-free dopamine, peroxidase (E.C 232-668-6), other redox enzymes, and inflammatory markers in both the striatum and cortical areas.

Materials and methods: Parkinson's disease was induced in CD21 mice through a dopaminergic lesion using 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) at a dosage of 25 mg/kg body weight, administered intraperitoneally (i.p.). The experimental groups included both MPTP-treated and control mice. After treatment, the whole brain was dissected, and the cortical and striatal regions were isolated for the analysis of dopamine, α-synuclein, and cytokines (IL-1α, IL-1β, IL-6, IL-10, and PGE2). Additionally, catalase, peroxidase, and glutathione reductase assays and kinetic studies with peroxidase-dopamine were performed. An AutoDock simulation was also conducted to explore the interaction between dopamine and peroxidase.

Results: The data showed significant decreases in IL-1β, IL-10, and PGE2 levels ($p < 0.05$) in the cortical area, while IL-1α decreased in both cortical and striatal regions ($p < 0.01$). IL-6 levels, however, increased in these brain regions. Peroxidase activity significantly decreased in the MPTP group ($p < 0.001$), whereas catalase activity increased in both cortex and striatum. These findings indicate a dysregulation of antioxidant enzymes in Parkinson's disease. Glutathione reductase levels also differed significantly ($p < 0.05$) between brain areas, with the most prominent changes in the cortex after MPTP treatment. While peroxidase-dopamine interaction was possible, no strong specificity was observed according to our *in silico* studies. Kinetic measurements demonstrated that dopamine acted as an uncompetitive inhibitor of peroxidase (E.C 232-668-6), explaining the reduced peroxidase activity *in vivo* after cell lesion by free contact not by concentration-dependent action.

Conclusion: The cerebral cortex appears to play a significant role in the biochemistry of Parkinson's disease (oxidative damage, dopamine reduction, α-synuclein accumulation, inflammation), which contrasts with much of the existing literature that emphasizes these changes as being more prominent in the striatum, traditionally considered the pivotal area in PD. While our findings require further experimental validation, they collectively suggest that the cortex may have a more critical role in PD than previously thought.

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Investigation of signaling pathways modulated by GPR27

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Introduction: GPR27 is an orphan receptor that belongs to the Super-Conserved Receptors Expressed in the Brain (SREB) subfamily of G protein-coupled receptors. This GPCR was reported to be involved in various physiological processes including energy metabolism, insulin secretion, and tumor progression. Several studies implied that GPR27 might play critical roles during cancer progression, particularly in hepatocellular carcinoma (HCC) [1], breast cancer [2] and glioma [3]. Our recent data demonstrated that human GPR27 with heterologous expression in HEK293T cells exhibits constitutive activity which proved to be G protein-independent but it is affected by the treatment of cells with isoproterenol (a well-known agonist of beta-adrenergic receptors). In the present work, we aim to investigate the implication of GPR27 and its potential ligand, isoproterenol in modulating Src kinase, respectively ERK1/2 signaling pathways that control cell growth, proliferation and migration. Our results will contribute to a better understanding of the mechanism underlying the involvement of GPR27 in cancer development and progression.

Materials and methods: HEK293T cells with heterologous expression of human GPR27 were stimulated or not with isoprotenerol and the phosphorylation levels of Src and ERK1/2 were detected by Western blotting. To identify key molecules involved GPR27-mediated signaling pathway, we performed aditional cells treatments with different compounds (protein kinase inhibitors, protein phosphatase inhibitors, FBS, growth factors, etc). Direct interaction between GPR27 and cSrc was investigated by immunoprecipitation followed by Western blotting analysis.

Results: Human GPR27 overexpressed in HEK293T determined a small increase of cSrc phosphorylation at Y419 site leading to an increase in Src activation in comparison with control cells, while the treatment with isoprotenerol generated a slight inhibition of cSrc phosphorylation at Y419 site, translated by a decrease in Src activity. On the other hand, GPR27 transiently expressed in HEK293T cells determined a rise in ERK1/2 phosphorylation signal which was further strengthened by the treatment with isoprotenerol.

Conclusions: Our results suggest that the constitutively active GPR27 modulates Src kinase and ERK-mediated pathways and its activity is affected by isoprotenerol. Further studies are needed to completely understand the molecular mechanism underlying GPR27 role in the development and progression of malignancy.

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Characterization of the Monomeric Hemoglobin From Invertebrates (*Chironomus sp.)*

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Introduction: To fully understand the plasticity of oxygen carriers and storage proteins, focusing solely on tetrameric hemoglobin is insufficient. In various aquatic environments, where oxygen availability fluctuates widely alongside parameters such as pH, salinity, and heavy metal concentration, larvae of *Chironomus* species synthesize monomeric hemoglobin. This hemoglobin is produced in response to hypoxia and is notable for its resistance to oxidative degradation and heavy metal effects [1]. Additionally, its ability to switch roles from an oxygen carrier to an oxygen storage protein has garnered significant interest [2]. Here, we present the reaction of *Chironomus* sp. hemoglobin with nitrite, its iron-dependent electrochemical potential, SDS-PAGE analysis, and PDB data recorded for this hemoglobin.

Materials and methods: The organisms were collected from an aquatic environment, and whole larvae were homogenized in PBS with a protease inhibitor cocktail. After centrifugation, the supernatant was stored at -80°C. Human hemoglobin (Hb) was used as the standard protein, with many laboratory data available for reference. Human Hb was isolated from red blood cells through repeated lysis, and its concentration was determined using the Soret band at 415 nm. The nitrite reaction was conducted with 5 μM Hb (both monomeric and tetrameric) previously reduced with sodium dithionite. The reaction was performed using three different nitrite concentrations (2, 5, and 10 mM) over 5 min. Differential pulse voltammetry (DPV) and cyclic voltammetry were applied to the same Hb concentrations following the electrochemical reduction of iron (III) to iron (II) and iron (0). SDS-PAGE was utilized to verify the Hb pattern, with the best results obtained using a 10% resolving gel. Monomeric Hb was also assessed in silico for the potential to form dimers spontaneously, with the results compared to those of tetrameric Hb.

Results: Chironomid hemoglobin (Hb) (Fig. 1) was found to be more resistant to nitrite compared to human Hb, with the catalytic rate (k_{cat}) being more significantly reduced in monomeric Hb than in tetrameric Hb. Electrochemical studies revealed two major aspects: (i) the reduction of iron in monomeric Hb occurred more slowly than in tetrameric Hb, and (ii) in monomeric Hb, iron was more immobilized compared to tetrameric Hb, resulting in lower heme availability. SDS-PAGE revealed an interesting aspect regarding protein subunits, as monomeric Hb appeared with two distinct spots in the denaturing system, despite theoretical and kinetic verification suggesting a single form. The spontaneous dimerization of monomeric Hb may explain this observation, though further studies are needed to confirm this hypothesis.

Conclusions: Animal monomeric hemoglobin (Hb) is noticed only in invertebrates, and the evolution from monomeric to tetrameric structures may be associated with a decrease in nitrite and oxidative stress resistance as hemoglobin transitions from a monomeric to a tetrameric form.

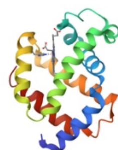

Fig. 1 Structure of monomeric Hb in different ligand states refined at 1.4 angstroms resolution (https://www.rcsb.org/structure/1ECA)

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Sequence optimisation for mRNA vaccines

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Introduction: Despite significant progress in recent years, mRNA vaccine development remains an active area of research for both expanding their use to new applications and identifying solutions to intrinsic problems of this development platform, such as stability, antigen expression and persistence *in vivo*, immunogenicity and reactogenicity. This study aims to evaluate the impact of synonymous sequence modifications of mRNA on the magnitude and duration of antigen expression *in vitro*.

Materials and methods: Using an in-house developed computer algorithm, the mRNA sequences encoding the green fluorescent protein (GFP) and the external region of the influenza virus hemagglutinin were optimized taking into account codon usage, the probability secondary structure formation in 3', maintaining an unstructured 5' region and reducing the probability of forming loops between 5' and 3'. Sequences optimized for different parameters and combinations thereof were tested on different cell lines and antigen expression was evaluated by fluorescence microscopy using the intrinsic emission of GFP and / or labeling with anti-His-tag or anti-HA-tag antibodies.

Results: While, taken individually, the different sequence optimization parameters had an inhibitory effect on antigen translation compared to the use of a randomly generated synonymous mRNA. However, simultaneous optimization of multiple parameters led sequences with increased antigen expression.

Conclusions: Sequence optimization is an important factor for controlling antigen expression in mRNA vaccines.

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Beyond the Membrane:
Nuclear Localization and Non-Canonical Signaling of GPR153 and GPR162

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Introduction: G-protein-coupled receptors (GPCRs) represent the largest family of transmembrane receptors, with over 1,000 genes coding for them, accounting for more than 1% of the human genome. In recent years, they have emerged as the most druggable receptors, with 40% of all FDA-approved drugs targeting only 10% of all GPCRs [1]. There is high interest in a special class of approximately 140 orphan GPCRs, for which their endogenous ligand is yet to be discovered. Among these orphan GPCRs are GPR153 and GPR162, which share 60% sequence similarity. These two receptors are highly expressed in the central nervous system and have broad pathological implications. Emerging evidence suggests that GPR153 plays a crucial role in the pathophysiology of neuropsychiatric disorders, including schizophrenia. Additionally, dysregulation of GPR153 expression is implicated in the progression of glioblastoma, indicating a potential role in tumorigenesis. On the other hand, altered expression of GPR162 has been linked to metabolic disorders such as obesity and diabetes, highlighting its significance in metabolic regulation [2].

Materials and Methods: HeLa cells were transiently co-transfected with human GPR153 or GPR162 cDNAs and specific biosensors for different signaling pathways. Intacellular cAMP levels were determined using the pGlo22F system (Promega). Serum response element (SRE) activity was measured using pGL4.33[luc2P/SRE/Hygro] (SRE-luciferase reporter gene assay)(Promega). Nuclear localization of both GPR153 and GPR162 was confirmed by immunofluorescence and Western Blots experiments on cellular fractions.

Results: Sequence analysis using the Moses Lab Prediction Tool(http://www.moseslab.csb.utoronto.ca/NLStradamus/) identified the presence of a Nuclear Localization Sequence (NLS) in the C-terminal domain of both GPR153 and GPR162. This NLS suggests a possible nuclear localization, which was experimentally confirmed by both immunofluorescence and Western blot. This atypical localization is a novel finding for Class A GPCRs, which are traditionally incorporated within the plasma membrane. This is not the sole aspect that doesn't confide within the canonical aspects of GPCRs. Both GPR153 and GPR162 induce a significant decrease in cAMP levels when transiently expressed into HeLa cells. This effect mimics a constitutively active Gi receptor but, intriguingly, this effect was not reversed by the Gi-specific inhibitor Pertussis Toxin. These results indicate that the mechanism by which these receptors regulate cAMP levels is distinct and independent of traditional G protein signaling pathways.

Conclusions: Both GPR153 and GPR162 exibit an atypical localization and signaling that set them apart from the typical Class A GPCR family. Further investigation is required to fully understand the molecular mechanisms underlying the nuclear localization and cAMP regulation of GPR153 and GPR162. Elucidating these pathways could provide new insights into their physiological roles and therapeutic potential in treating related diseases.

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GPR27: Breaking the Norms of Conventional GPCR Signaling

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Introduction: G protein-coupled receptors (GPCRs) are crucial membrane proteins involved in regulating various cellular and physiological processes. They are highly successful targets for drug development, with 30% of FDA-approved drugs acting on them. However, in humans, 160 of these receptors remain with unidentified functions and are classified as orphan GPCRs. Among these, the Super-conserved Receptors Expressed in Brain (SREB) family includes three orphan receptors: GPR27, GPR85, and GPR173. GPR27 has been associated with increased insulin production, suggesting a potential role in type II diabetes [1]. Additionally, hypermethylation of CpG sites in the GPR27 gene indicates a possible involvement in cancer [2].

Materials and methods: HEK293T cells were co-transfected with human GPR27 and specific biosensors for different signaling pathways. cAMP levels were determined using the pGlo22F system (Promega). Serum response element activity was measured using pGL4.33[luc2P/SRE/Hygro] (SRE-luciferase reporter gene assay). β-arrestin2 recruitment was assessed by firefly luciferase complementation (a gift kindly provided by Dr. Julien Hanson, Liege University). Expression of GPR27 in INS1E cells was deternined by qPCR. Insulin secretion was measured by Homogeneous Time Resolved Fluorescence (HTRF) (Cisbio). **Results:** When heterologously expressed in HEK293T cells, human GPR27 receptor induces cellular effects that resemble a constitutively active state of the receptor in a G-protein-independent manner. Through comprehensive cellular analysis of GPR27-dependent modulation of transcription factors, we identified an adrenergic ligand, namely isoproterenol which specifically inhibits serum response element (SRE) activity. Notably, this inhibition was resistant to G-protein inhibitors and independent of beta-arrestin, as confirmed by a beta-arrestin recruitment assay. Further experiments revealed that isoproterenol markedly suppressed epidermal growth factor (EGF)-induced SRE activation in a concentration- and receptor-dependent manner. Additionally, knockdown of GPR27 in INS1E cells using siRNA resulted in enhanced glucose-stimulated insulin secretion.

Conclusions: GPR27, a possible novel adrenergic receptor, displays a unique signaling behavior that deviates from typical Class A GPCR family. Gaining a deeper understanding of its signaling pathways is crucial for uncovering the potential role of this novel molecular target in diabetes and possibly cancer.

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Exploration of Tau protein auto-acetylation mechanism

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Introduction: Most posttranslational modification sites of intrinsically disordered Tau protein are located within the microtubule-binding region (MTBR) and can interfere with its physiological function. Among the putative pathological processes underlying neurodegenerative diseases, lysine acetylation of neuronal Tau protein has emerged as a key modification and has thus become a topic of extensive research [1]. Furthermore, it has been shown that Tau protein has an intrinsic ability to auto-acetylate itself, indicating an intricate pattern of overall acetylation [2].

Materials and methods: Experiments were performed using a truncated form of Tau protein (K18) containing the 4 repetitive regions known to form the MTBR, both wild type and a Cys-to-Ala double mutant, which is predicted to lack the ability to auto-acetylate. The proteins were expressed prokaryotically and further purified by ion exchange chromatography. Acetylation over time (in the presence or absence of acetyltransferase) was monitored by Western blotting with specific antibodies. The interaction between Tau protein and the acetylation agent acetyl-coenzyme A (AcCoA) was assessed by a pulldown assay using its backbone, Coenzyme A (CoA), as the immobilized interacting partner. Next, 2 models were generated using the AlphaFold server; of the generated models the highest ranking was used. After generation, the models were minimized using OpenMM. The generated models were parametrized using the ff19SB protein forcefield and conformational sampling was performed. CoA was parametrized using General Amber Force Field 2 (GAFF2). The Robosample software suite was used to run molecular dynamics simulations [3].

Results: Both truncated Tau proteins were obtained with high yields and satisfactory purity after optimization of prokaryotic expression and subsequent purification steps. As expected, the wild-type protein showed a gradually increasing (auto)acetylation tendency over time, even at early reaction points, unlike the Cys-to-Ala double mutant that remained unmodified. Interestingly, both proteins showed a persistent interaction with CoA even at long incubation times, supporting the main hypothesis of an auto-acetylation mechanism. Moreover, molecular docking simulations indicated preferential binding sites for CoA on the unfolded truncated forms of Tau protein.

Conclusions: This study offers valuable insights into the auto-acetylation process of Tau protein, and our results strongly suggest a complex interplay of different MTBR residues in this process, which requires further investigation.

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The quality of pork burgers enhanced by the extract of *Prunus serotina* fruits.

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Introduction: Fortification of the meat stuff with plant origin compounds is one of the most recent trends in meat processing. The role and fortification paths are very diverse and cover multiple requirements, from nutritional fortification, protect adulteration, improve sensory parameters and oxidative stability [1]. Our research was focused on using a new and relative rare plant in Romania – *Prunus serotina* – to prepare a natural concentrate that is suitable for fortification of meat products (pork burgers). Our research focused on the use of a less cultivated plant in Romania, *Prunus serotina*, to prepare a natural concentrate that is suitable for fortifying meat products (pork burgers). The effect of different concentrations of *P. serotina* extract on the nutritional composition, the total phenols content, antioxidant capacity and color parameters (L*a*b*) of the burgers was followed before and after cooking.

Materials and methods: The *P. serotina* fruits extract was obtained by maceration in ethanol (1:1, w/v) at 20°C. Following the ethanol's removal using a rotary evaporator, the resulting extract (PSE) was characterised using physico-chemical parameters (pH, Brix), the Folin-Ciocalteu assay for total phenol concentration, and the DPPH assay for antioxidant capacity. The various PSE concentrations were added to pork burger pastilles and evaluated based on the amount of protein and fat, in accordance with the regulations (SR ISO 937:2007 and SR ISO 1443:2008, respectively). Using an Agilent 7890 Gas Chromatograph fitted with a Flame Ionization Detector, the fatty acid profile of pork burgers with and without PSE was determined. An imagistic approach based on CIELAB was applied to assess color of burgers enriched with PSE.

Results: The BRIX value of the PSE extract was 17.75 ±0.07, and its pH was 4.28 ±0.02. PSE's antioxidant capacity was measured at 6.72±0.69 mmol TE/ml, and its total phenolic content was 1.85 mg GAE/ml. Pork burgers' nutritional value (proteins and fats) remained unaffected by the addition of PFA, while the fatty acid composition changed slightly ($p>0.05$) based on the extract's concentration. Burgers' bioactive component content increases as PSE levels rise, and consequently their antioxidant capacity. The brightness parameters (L*) decrease as the concentration of PSE increases, whereas the red-green coordinate (a*) increases. Following cooking, the antioxidant capacity and phenols content decreases.

Conclusions: The PSE added as natural aditive increase the product sensory properties such as color. Also the structure of fatty acids recorded very specific evolution according with fruit concentrate addings.In the conclusion the use of PSE is an viable alternative for the reducing the synthetic additives in the case of pork burger.

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Hsa-miR-19a/b as levodopa therapy responder in Parkinson's disease

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Introduction: Parkinson's Disease (PD) is characterized by the progressive loss of dopaminergic neurons due to environmental and/or genetic mechanisms []. In spite of the decades-long efforts, none of the research aiming to define PD diagnostic and LevoDOPA (LD) therapy monitoring biomarkers were able to make the transition from bench to clinic. Among these candidates, microRNAs (single-stranded, small RNAs post-transcriptionally regulating the expression of target genes, including those involved in PD pathogenesis) are known for their remarkable stability in biological fluids. Previously, we used semiquantitative RT-PCR to characterize hsa-miR-19a and hsa-miR-19b plasma expression in PD patients and showed that hsa-miR-19b is dose-dependently expressed in cultured dopaminergic neurons and hippocampus of adult mice exposed to LD [].

Materials and methods: Here we measured the absolute number of plasma hsa-miR-19b and examined the degree of correlation with plasma LD, the demographic and clinical variables of PD patients.

Results: Absolute quantification shows that plasma hsa-miR-19a and hsa-miR-19b expression levels are strongly correlated (in both LD-treated and naïve PD patients) and are differentially expressed in naïve PD patients after LD therapy initiation. The two microRNAs correlate with the rest tremor amplitude in PD patients under therapy but not in naïve patients. Plasma hsa-miR-19a and hsa-miR-19b levels also correlate with the gastrointestinal pathology and plasma cholesterol levels in PD patients under LD therapy but not in naïve patients.

Conclusions: Plasma hsa-miR-19a and hsa-miR-19b are putative LD responders and might serve as biomarkers for monitoring the clinical response to LD therapy in PD patients. Further research is needed to quantify the impact of gastrointestinal pathology on the accuracy of hsa-miR-19a and hsa-miR-19b plasma levels in the context of PD pathology.

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Differences in 3p/5p miRNA quantification are independent of the microRNAs' GC content and secondary structure stability

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Introduction: The canonical synthesis of microRNAs involves two steps catalyzed by the endonucleases Drosha and Dicer. Once synthesized, the guide strand is uploaded into the microRNA-Induced Silencing Complex, while the passenger strand is degraded. Thus, the ratio between the two (irrespective of their 3p or 5p origin of the pre-miRNA) is in favor of the former. However, increasing evidence suggests that both the guide and passenger strands have the potential to be biologically active. The selection of a small RNA purification method and qRT-PCR platform, along with microRNA GC content and secondary structure stability, play a crucial role in measuring microRNA levels and determining the guide and passenger strands[1].

Materials and methods: Here, we used one of the most frequently used purification technique (QIAGEN miRNeasy Serum/Plasma kit) purify several 5p/3p synthetic microRNAs with various GC contents from mixtures with MS2-RNA carrier. Next, we used dedicated inventoried TaqMan assays to build calibration curves that were subsequently used for absolute quantification.

Results: We documented statistically significant differences between the -3p/-5p miRNAs at concentrations relevant to biological activity. These differences depend on the strand concentration but are independent of their GC content and of their Homodimer, Heterodimer, and Monomer Ensemble Free Energy.

Conclusions: Our data suggests that purification/quantification techniques might significantly influence 3p vs 5p microRNA quantification with a notable impact on the definition of guide vs. passenger strands. This finding has implications for future research in arm switching, and the development of accurate microRNA quantification methods.

Plasma hsa-miR-19b response to immunotherapy in Non-Small Cell Lung Cancer patients

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Introduction: Non-Small Cell Lung Cancer (NSCLC) accounts for 80% of lung cancer cases, making it the primary cause of cancer-related deaths worldwide. The treatment of NSCLC involves immune checkpoint inhibitors targeting PD-1, PD-L1, and CTLA-4, which boost the immune system's activity against cancer cells. Nevertheless, only 20-30% of NSCLC patients show positive results. Currently, PD-L1 expression and tumor burden are the sole established indicators (30% accuracy) for immunotherapy effectiveness in NSCLC patients. Hence, there is a critical requirement for more precise, easily accessible biomarkers to forecast the immunotherapy response []. MicroRNAs are small noncoding RNAs involved in posttranscriptional gene expression regulation. Due to their exceptional stability in biological fluids, they are seen as ideal noninvasive diagnostic and prognostic biomarkers.

Materials and methods: In this study, we used absolute quantification by qRT-PCR to evaluate changes in plasma hsa-miR-19b-3p levels during anti-PD-L1 therapy in advanced NSCLC patients. Plasma levels of hsa-miR-19b-3p were compared to patient response data, clinical and paraclinical variables throughout immunotherapy.

Results: Our findings indicate that plasma levels significantly decrease as soon as three months after the onset of immunotherapy. The change in plasma remains stable throughout the course of immunotherapy and correlates with the demographic and clinical variables of NSCLC patients. Bioinformatics analysis utilizing miRWalk prediction and STRING complex network analysis suggests a potential functional connection between hsa-miR-19b-3p and the PD-L1 signaling pathway in NSCLC.

Conclusions: Plasma level of hsa-miR-19b-3p may be utilized in constructing a predictive risk nomogram based on a multivariable prognostic model to classify patients early on as either responders or non-responders to immunotherapy.

Establishing specific chiral based separation methods for polypeptides with different side chains

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Introduction: Chiral separations are challenging methods with application in various fields such as pharmaceutical, medicinal chemistry and biology. The chiral compounds have asymmetry at the atom of carbon, sulfur, phosphorus or nitrogen etc. The optical isomers may have different effect or can have different degrees of efficacity. That's why it might be necessary to separate the isomers using a chiral chromatographic technique [1]. The chiral compounds can possess asymmetry at the atom of carbon, sulfur, phosphorus or nitrogen etc. In was observed that in the case of peptides oxidized the sulfur atom of the methionine the racemic sulfoxides can have different biological properties compared to unoxidized ones at methionine [2]. Our aim was to separate stereoisomers of these molecules that could show distinct biological properties. Here we report the analysis of peptides containing at least an oxidized Methionine residue using liquid chromatography coupled with UV-Vis and CD detection.

Materials and methods: Various peptides containing oxidized methionine residues were analyzed using reverse phase liquid chromatography. The chiral stationary phases were derived from cellulose. The chromatographic experiments were performed with a JASCO HPLC equipped with a quaternary pump PU-2089 plus and a circular dichroism detector CD-2095 plus. In some cases, the absolute configuration was attributed to each optic isomer.

Results: We obtained distinct resolutions using various experimental conditions. It was observed that enantioselectivities increased when using acidic modifiers, but the reproducibility was poor. The selectivity decreased to zero after several injections. We also tested chaotropic agents dissolved in the aqueous mobile phase and we obtained satisfactory results.

Conclusions: The results demonstrate that the methodology can be suitable for the isolation and purification of stereoisomers with purity higher that 90%. In the future we aim to extend our workflow in order to include other biological peptides.

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Innovative treatment based on non-steroidal anti-inflammatory drug (NSAID) formulations for chronic diseases

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Introduction: Rheumatoid arthritis (RA) is known as a chronic autoimmune inflammatory disease, with a great risk of development as advancing in age and with a continuously increasing global burden. It manifests as a symmetrical polyarthritis, characterized by chronic pain, stiffness, and swelling of any joints [1,2]. Moreover, individuals with RA exhibit elevated oxidative stress levels [3]. Conventional synthetic disease-modifying antirheumatic drugs (DMARDs), such as methotrexate, are commonly used as first line treatment in RA [2,4]. Furthermore, non-steroidal anti-inflammatory drugs (NSAIDs), having analgesic and anti-inflammatory activity, significantly reduce pain and stiffness, while not causing joint damage and disease modification [1,3,5]. The present study explores the effect of NSAID-BSA conjugates compared to the commercialized forms in collagen-induced-arthritis (CIA) rat models. Based on our discoveries, NSAID conjugates can be potential candidates for rheumatoid arthritis management.

Materials and methods Treatment with standard ibuprofen, standard diclofenac, and the conjugated forms (10 mg/kg) were administered orally, daily for fourteen days. The experiment was terminated 28 days after the first injection, animals were sacrificed, and samples were collected for hematologic, biochemical and histopathologic examination.

Results: Our results showed that ibuprofen and diclofenac conjugates expressed similar activity to the standard drugs, while having significantly reduced toxicity compared to the parent drugs, as seen in the levels of interleukines (IL-6, IL-2), TNF-alpha, oxidative stres markers and antioxidant enzymes.

Conclusions: . Based on our discoveries, NSAID conjugates can be potential candidates for chronic diseases such as rheumatoid arthritis.

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SKOV3 actin-GFP ovarian cancer stable cell lines for 4D imagistic analysis of cancer cell processes upon treatment with chemo- and FLASH radiotherapy

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Introduction: Ovarian cancer metastasis is the most fatal gynecological malignancy in the developed world, as most diagnoses are given in advanced stages and less than 29% patients have a survival rate of 5 years [1]. Recently, radiotherapy at ultrahigh dose rates has shown promising potential to be used as a novel therapeutic strategy for cancer treatment. In our recent studies [2] we have shown for the first time that FLASH ionizing radiation produced by very high energy electrons generated by a high-intensity laser plasma accelerator system can produce differential effect on melanoma cancer cells and normal melanocytes. Herein, we further propose FLASH radiotherapy for "deep-seated" ovarian cancers which cannot be treated with conventional therapy, as they affect the abdominal cavity. We anticipate better understanding of FLASH *in vitro* effect in comparison with conventional radiotherapy and chemotherapy by using advanced real time imaging tools for studying cell behavior upon radiation exposure.

Materials and methods: For our studies, we employed SKOV3, an ovarian cancer cell line known to be resistant to chemotherapy (cisplatinum, adriamycin) and radiotherapy [3]. Additionally, we used the knock out (KO) SKOV3 cell line where tissue transglutaminase (TG2) – a marker of resistance to chemo- and radiotherapy – was excised. In order to analyze SKOV3 ovarian cancer cells in co-culture with other cells and to explore the cancer cell behavior by live cell imaging upon treatment with either chemotherapy drugs or radiation, we obtained actin-GFP SKOV3 stable cell lines. First, we transduced SKOV3 Wild Type (WT) and TG2KO cell lines with LifeAct-14 plasmid (LA) (#158750, addgene) so as to express stable actin-GFP. Then, we expanded the cells and selected them with 5 μg/mL blasticidin. Further on, we selected the GFP^+ clones using FACS AriaIII cell sorter. Next, we performed an enrichment experiment in which we sorted the cells with high (HIGH LA) and low expression (LOW LA) of GFP, respectively.

Results: We have confirmed the actin-GFP expression of SKOV3 WT and TG2KO stable cell lines by FACS diagrams and fluorescence microscopy. We also observed cell motility in time-lapse microscopy experiments of parental cell lines HIGH LA *vs.* LOW LA GFP cells obtained after enrichment.

Conclusions: We have obtained fluorescently-labeled ovarian cancer cells to be used for advanced 4D imagistic analysis by time lapse investigation after exposure to FLASH radiation. Further experiments are dedicated to quantitative measurements of cell motility on SKOV3 WT and TG2KO actin-GFP HIGH LA and LOW LA GFP^+ cell lines to determine the most suitable cell line model to be used in our radiobiology studies by live-cell imaging.

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A multiomic analysis of nicotine degradation via the pyridine pathway in *Paenarthrobacter nicotinovorans* ATCC 49919

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Introduction: *Paenarthrobacter nicotinovorans* ATCC 49919 uses the pyridine pathway encoded by the pAO1 megaplasmid to degrade toxic nicotine and produces non-toxic chemicals of economic importance. The strain could become a key biological agent for nicotine decontamination from waste or polluted natural resources. Unfortunately, little is known about the interactions between the metabolic pathways encoded by the chromosome and pAO1. Additionally, there is no experimental evidence of expression during nicotine catabolism of half of the 40 genes encoded by the pAO1 megaplasmid that are putatively involved in nicotine catabolism (*nic* genes). Furthermore, the nicotine transporters and transcription factors controlling nicotine degradation are unknown for this species. Therefore, to improve our understanding of microbial nicotine degradation via the pyridine pathway and facilitate the development of biotechnological applications of *P. nicotinovorans* ATCC 49919, we aimed to bridge the gaps between the available biochemical, biomolecular, proteomic and genomic data by performing a multiomic study.

Materials and methods: *P. nicotinovorans* ATCC 49919 was grown on citrate medium in the absence and presence of nicotine. Using ultra-performance liquid chromatography, three key time points of nicotine degradation in *P. nicotinovorans* ATCC 49919 were established. These are representative for: the onset, slowing and cessation of nicotine catabolism and respectively correlate with the early log phase, the late log phase and the late stationary phase. Bacterial cells were sampled in replicate at the three key time points; total RNA was isolated and used to prepare libraries for direct long-read sequencing. To identify the most differentially expressed genes and enriched pathways in the presence of nicotine at the three key time points of nicotine catabolism in *P. nicotinovorans* ATCC 4991, the transcriptomic raw data was bioinformatically processed and integrated with a previously available proteomic dataset which we re-interpreted using the recently available complete genome of the studied strain.

Results: The generated transcriptomic data provided the first experimental evidence of the *in vivo* expression of 11 of the 40 *nic* genes. In addition, we identified and described for the first time several chromosomal genes whose expression significantly varied (p-adj < 0.1) under nicotine supplementation. Gene-set and pathway enrichment analyses revealed that the most differentially expressed genes in the nicotine-treated cells are involved in transport, growth and metabolism, plus resistance to the oxidative and osmotic stress caused by nicotine and its derivatives. In addition, we proposed a series of genes which likely encode transcription factors that control nicotine degradation and the cell's response to the catabolic process. The transcriptomic data supports and confirms a previously formulated hypothesis based on the proteomic analysis: the final products of nicotine catabolism are integrated into the citrate cycle, the nicotine catabolic pathway being anaplerotic in *P. nicotinovorans* ATCC 49919.

Conclusions: This is the first multiomic analysis of a microorganism that degrades nicotine using the pyridine pathway. The transcriptomic data confirmed and supplemented the previously obtained and newly reinterpreted proteomic information, providing the first evidence for the expression and function of several megaplasmid and chromosomal genes during nicotine catabolism. The integrated data revealed several complex molecular mechanisms that are employed by *P. nicotinovorans* ATCC 49919 to compensate the oxidative and osmotic stress induced by nicotine catabolism.

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A compensatory research study to evaluate the impact of 3D-printed molecular models on teaching protein and DNA structure

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Introduction: The structure-to-function relationship is the hallmark of biochemistry and molecular biology. Although very useful and convenient, the two-dimensional structural formulae that students usually first encounter in the chemistry class are not able to picture the complexity of macromolecular structures such as proteins and nucleic acids. For this, animations, movies, and virtual reality are much better suited. Still, touching and handling physical models of molecules should allow students to better overcome the problems associated with the translation of 2D formulae into 3D space. Object-based learning stands out as an approach that gives students a tangible way to view and manipulate physical structures in three dimensions, strengthening learning by providing a more complete sensorial experience and challenging students to engage with and interrogate the object. To test this hypothesis, a compensatory research design was employed.

Materials and methods: Second-year bachelor students enrolled in the Molecular Biology class were randomly allocated to two groups. Both groups attended independently two lectures and were alternatively control and intervention groups. In the control group, only animations and drawings were used while in the intervention group, the same animations and drawings were replaced by 3D printed molecular models of various amino acids and nucleotides, peptides, a-helices, b-sheets, proteins and DNA in various representations [1]. Models were used by the educator in front of the class but also handed to the students who were given time to interact with the models. Before and after each lecture, both groups received the same pre- and post-test consisting of a total of 23 questions evaluating key biomolecular visualization learning goals [2]. At the end of the experiment, the students were asked to fill in an anonymous feedback form.

Results: Presenting the physical molecular models in the class and allowing students 3-5 minutes to handle them individually or in small groups was shown to be enough to convert low gain lectures (mean g_{ind} around 0.2) into medium gain lectures (mean g_{ind} around 0.4). The physical models were received by students as being helpful because it allowed them to better focus, to engage the visual memory and because it provided a hands-on advantage. Despite some identified drawbacks, the usage of physical models of molecules fabricated using 3D printing is great way of improving bio-molecular education with low costs, including in low-income countries where teaching materials are scarce.

Conclusions: 3D printed molecular models have been integrated into two lectures dealing with DNA and protein structure. The impact of the models on students' ability of overcome some common misunderstandings related to proteins and DNA structures was evaluated in a randomized controlled experiment. The current work contributes to the body of empirical evidence that support the use of 3D representations in teaching and learning biochemistry concepts.

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Development of a biotransformation assay for converting nicotine into 6-hydroxy-L-nicotine using *Paenarthrobacter nicotinovorans* NCAIM P(B) 001499

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Introduction: 6-hydroxy-L-nicotine (6HLN), the first metabolite in the nicotine degradation pathway of *Paenarthrobacter nicotinovorans* pAO1 ATCC 49919, improves memory and behavioural responses by reducing oxidative stress in the brain. Since the degradation of nicotine via the pyridine pathway in *P. nicotinovorans* pAO1 ATCC 49919 does not stop at 6HLN, the technical challenge in obtaining this compound through bioconversion with bacterial cells is to identify an efficient method to reduce or stop its conversion to the next compound, 6-hydroxy-pseudooxynicotine. The strategy involves increasing the expression of the enzyme nicotine dehydrogenase (NDH) and reducing the conversion of 6HLN to 6-hydroxy-pseudooxynicotine by applying chemical inhibitors of 6-hydroxy-L-nicotine oxidase (6HLNO) like $ZnSO_4$. *P. nicotinovorans* strain NCAIM P(B) 001499 - derived from *P. nicotinovorans* pAO1 ATCC 49919 and overexpressing the enzyme NDH have been developed in our lab for this purpose [1]. This study aims to apply a biocatallisis sistem similar similar to the ones described for *Pseudomonas sp. HZN6* and *Agrobacterium tumefaciens* S33 [2] [3] but on *P. nicotinovorans* NCAIM P(B) 001499 in order to improve the production yield of 6HLN.

Materials and methods: Either *Paenarthrobacter nicotinovorans* pAO1 ATCC 49919 (wild type reference strain) or *P. nicotinovorans* NCAIM P(B) 001499 was grown for 12-hours on citrate medium supplemented with nicotine, 190 rpm and 28°C. The nic-induced cells in log phase were recovered by centrifugation at 4,500 rpm for 20 minutes and washed three times with 20 ml of sterile distilled water. The amount of cells obtained is weighed and resuspended in a 1:1 ratio in sterile distilled water (1 ml: 1 g of cells). The biotransformation assay was performed in water by incubating the nic-induced log-phase cells for up to 7 hours at 28°C 190 rpm with 3 mM nicotine in a 50 ml of sterile tube. At regular intervals 1 ml of supernatant is collected and HPLC analysis on a Shimadzu Prominence UPLC system equipped with a RP-18e 150-4.6 mm HPLC column (Chromolith HighResolution Sorbet Lot/Column No. U1141/010). The mobile phase was a mixture of 1 mM H2SO4: methanol (90:10 v/v) at a flow rate of 1 ml/min. The separation was performed at 30 °C using isocratic elution and 6HLN levels were monitored at 290 nm.

Results and conclusion: The wild type *Paenarthrobacter nicotinovorans* pAO1 ATCC 49919 was used as reference and 0.2 to 5 grams of biocatalyst were tested. These base-line preliminary tests showed that 6HLN accumulated earlier when more biocatalyst is used, but also that 6HLN was much faster consumed by the cells – both nicotine and 6HLN are depleted after 250 min when using either 0.5, 1 and 5 g of cells in 50 ml assay mixture. When *P. nicotinovorans* strain NCAIM P(B) 001499 cells overexpressing the NDH gene were used as biocatalyst, the nicotine consumption rate increased, but 6HLN persisted in the assay medium for longer and at higher levels when using 0.5 and 1 g biocatalyst/50 ml assay. Amounts higher the 1 grams speed up the conversion reaction to bellow 50 min and increased the maximum of 6HLN accumulation in the medium. Data is still processed for the calculation of conversion rates and yield.

Conclusion: A method for preparing a biocatalyst based on *P. nicotinovorans* strains useful for the biotransformation of nicotine into 6HLN has been established. Some preliminary data on the amount of biocatalyst required for quick bioconversion is available and further investigations on the best substrate concentration and use of various enzyme inhibitors are under way.

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Recovering a lost sequence – the pH6EX3 cloning and expression plasmid

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Introduction: The pH6EX3 expression vector directs the synthesis of a fusion protein with an N-terminal 6-His tag, simplifying the purification of the overexpressed protein through IMAC. Derived from pGEX-2T, pH6EX3 provides very efficient protein overexpression. Despite its widespread use since its publication in 1992, pH6EX3 sequence was not deposited to any publicly available databases and has been lost. Hence, our aim is to recover and share its sequence with the scientific community. We used nanopore sequencing to assemble the pH6EX3 sequence and add it to Addgene.

Materials and methods: Plasmid DNA was extracted from bacteria cultured on LB medium (50 µg/ml ampicillin) and was isolated with the Qiagen Plasmid Miniprep kit. Library preparation was done with the ONT SQK-RAD004 kit followed by the long-read sequencing using an ONT MinION device coupled with a Flongle flowcell. After basecalling with Guppy 6.5.7, the epi2me-labs/wf-clone-validation pipeline was employed for QC, assembly, and annotation.

Results: A total of 1129 reads were obtained, with a median length of 4805 bp and a median Q-score of 10. The circular plasmid was accurately assembled, achieving an average quality score of 33.55 and a final size of 4856 bp. All sequence features outlined in the original publication were successfully identified by pLannotate.

Conclusions: Long-reads sequencing allowed the almost-complete sequencing of the pH6EX3 plasmid in a single read. The circular plasmid sequence and its features closely match those described in the original report. The sequence is available in Addgene, Plasmid #216723 (https://www.addgene.org/216723/) and can be used for cloning and purification of various recombinant proteins in Escherichia coli strains.

The Role of Two-Pore Channel Proteins (TPCs) in Colorectal Cancer and Inflammatory Bowel Disease: Insights from a Romanian Patient Cohort and the Characterisation of TPC2 in IBD Pathophysiology

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Introduction: Inflammatory Bowel Disease (IBD) is associated with an increased risk of dysplasia and colorectal cancer (CRC). Two pore channel proteins (TPC) are endosomal/lysosomal ion channels that mediate calcium release, triggered by the second messenger, nicotinic acid adenine dinucleotide phosphate (NAADP). The NAADP/TPC Ca^{2+} signalling pathway has been demonstrated to play key roles in T cell activation, which is central to the pathogenesis of IBD and in cancer cell proliferation and survival. A predominant Th1 cytokine response profile has been identified in both Crohn's disease (CD) patients and mouse models of colitis with increased inflammatory cytokines levels, delayed type of hypersensitivity and granulomatous inflammatory reaction. Here, we assessed the expression levels of total TPCN1 and TPCN2 mRNA in a cohort of Romanian patients diagnosed with CRC. Additionally, explored the contribution of TPCN2 in the dextran sodium sulphate (DSS) model of colitis on inflammation in the *Tpcn2*$^{-/-}$knock-out mouse.

Material and Methods: The role of TPC2 in CD was studied in six CD patients during clinical remission after corticosteroid treatment. Double immunofluorescence staining, high-resolution deconvolution microscopy, and co-localization of anti-TPCN2 and anti-lysosomal associated membrane protein (LAMP) antibodies were performed on lesional and control histopathological tissue sections. We also analysed 25 CRC patients who underwent surgery, collecting both tumour (T) and peritumoral (PT) mucosa for a two-step RT-PCR assay. *Tpcn2*$^{-/-}$ mice, which show no overt phenotype or gastrointestinal abnormalities, were treated with DSS to induce colitis, and samples were collected for gene expression and histopathological analysis.

Results: Histopathological analysis of CD patient biopsies showed significantly increased LAMP2 staining (p=0.02) and decreased TPCN2 staining (p=0.02) in inflamed tissue compared to non-inflamed tissue. TPCN2 expression increased gradually from inflamed to unaffected regions, with a significant LAMP2/TPCN2 ratio difference between inflamed and non-inflamed tissues (p=0.039). In CRC samples, TPCN1 and TPCN2 expression was higher in non-invaded PT tissue in 68% and 60% of cases, respectively, with significant differences between tumour and PT tissues (p=0.005 for TPCN1, p=0.001 for TPCN2). There was a strong correlation between TPCN1 and TPCN2 expression in tumour and PT tissues (p<0.0001). In the *Tpcn2*$^{-/-}$ DSS-treated mice, colonic tissue showed severe goblet cell depletion, epithelial cell loss, hyperplasia, crypt loss, oedema, and crypt abscesses, with over 50% of the colon affected compared to control mice.

Conclusions: Our data suggest that alterations in acidic-store Ca^{2+} release may predispose individuals to IBD, as indicated by increased LAMP2 and decreased TPCN2 expression in CD biopsy samples. In CRC patients, elevated TPCN1 and TPCN2 expression in non-invaded PT tissue suggests these genes could serve as independent prognostic markers. Furthermore, our murine model demonstrates increased susceptibility to DSS-induced colitis in the absence of TPC2. These findings highlight the potential of targeting TPCs as a novel therapeutic strategy for IBD and CRC, warranting further investigation into their roles and utility as biomarkers for disease prognosis and therapy.

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Sequence based bioinformatics analysis of 61 plasmids related to pAO1 megaplasmid of *Paenarthrobacter nicotinovorans*

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Introduction: pAO1 megaplasmid of *Paenarthrobacter nicotinovorans* is a conjugative catabolic megaplasmid. It has a modular structure, consisting of a backbone with genes for plasmid core functions and one gene cluster responsible for nicotine degradation that was acquired by horizontal gene transfer. pAO1 is used as a model for studying the molecular evolution of nicotine catabolic pathway and its spread among related genera of soil nicotine-degrading bacteria: *Paenarthrobacter*, *Arthrobacter*, *Rhodococcus* and *Nocardioides*. This study performs a comparative analysis of all the available plasmids from these genera, in an attempt to shed light on the genetic diversity and similarity of these plasmids and find to the most genetically similar to pAO1.

Methods: Sequences of 61 complete and circular plasmids of soil nicotine-degrading bacteria and have been identified and downloaded from Genbank on 25.10.2023. dDDH values were calculated using GGDC 3.0 and ANI values with OrthoANI. Data was visualized with Heatmapper where hierarchical clustering was performed by average linkage and Euclidean distances were calculated.

Results: dDDH values from the pairwise comparisons of the 61 plasmids allowed us to identify one major cluster containing 9 plasmids with highly similar sequences. These plasmids belong to *Arthrobacter* and *Paenarthrobater* strains and are similar with pAO1. A second smaller cluster containing plasmids pA, pB, pC from *P. urefaciens* strain AT, pADNL1 from *P. urefaciens* strain DnL1-1, and pTC1 from *P. aurescens* strain TC1 can also be described. ANI data shows the existence of two major plasmid clusters – one contains plasmids belonging to *Arthrobacter/Paenarthrobacter*, and another belonging to *Rhodococcus* strains.

Conclusions: Knowing that the two parameters are calculated differently: dDDH is calculated using the full DNA sequences of the plasmids, while ANI is calculated using only orthologues genes, the slight difference in clustering is expected. Still both heatmaps indicate the existence of a core set of common genes for *Arthrobacters/Paenarthrobater* and a different core set for *Rhodococus*. Also, the dDDH heatmap indicates two different plasmid lineages in *Arthrobacter* and *Paenarthrobater*, where the pAO1 lineage is the most abundant.

Design of a simple, inexpensive SERS substrate optimized for rapid detection of nucleic acids

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Introduction: Surface-Enhanced Raman Spectroscopy (SERS) is becoming an essential analytical technique due to its efficiency, simplicity, and speed. It offers real-time, non-destructive, ultrasensitive detection of target analytes of interest with extremely low detection limits. The main challenge is developing substrates that are reproducible, cost-effective, and efficient. In this context, we design a new plasmonic paper-based nanoplatforms with interesting capabilities in terms of sensitivity and reproducibility for promoting rapid detection of target analytes via Surface Enhanced Raman Spectroscopy (SERS).

Materials and methods: Two different shapes of gold nanoparticles, i.e. nanospheres and nanorods as highly active plasmonic nanotransductors with different optical responses, were firstly synthesized in colloidal solution, and then immobilized onto a flexible and inexpensive Whatman paper. The immobilization protocol of these two different nanoparticles shapes by simply manipulation of their optical density directly on paper were rigorously investigated.

Systematic measurements were conducted to assess the effect of GNP concentration on the SERS signal intensity of 4-MBA, used as Raman probe. Then we validated our flexible nanoplatforms using different nucleic acids such as PolyA, PolyT and PolyU.

Results: Systematic measurements have shown that the SERS signal increases as a function of nanoparticles concentrations. In addition, we were also able to detect nucleic acids using the portable Raman spectrometer using this SERS substrate.

Conclusions: This study demonstrated the simplicity and low cost of this SERS substrate for rapid and efficient nucleic acid detection using a miniaturized, portable Raman spectrometer. While the non-homogeneous structure of these substrates can lead to variability in signal reproducibility, they are still advantageous for fast, affordable, and portable analysis across various applications.

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Cell behaviour on novel dental implant-abutment titanium-based modified interfaces

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Introduction: Dental abutments are essential components of dental prosthetics, serving as connectors between implants and prosthetic devices. Epithelium attachment to the abutment surface plays a critical role in the success of dental implants preventing tissues and bone pathogen infection. The integration of dental abutments with surrounding tissues is influenced by its surface characteristics and the interaction with various cells, primarily involving mesenchymal stem cells (MSC), epithelial (GEpi), and fibroblast (GF) cells. Ultrafast laser processing technique is an alternative tool for surface improvement of dental abutments and fabrication of nanostructures on large scale [1]. Here we investigated the interactions between titanium (Ti)-based laser modified surfaces and cell components of the gingival and bone tissue [2,3] which are crucial for preventing the peri-implant disease and implant failure and to increase implant life-time.

Materials and methods: The titanium and titanium-based alloy surface modifications were generated by high-repetition rate picosecond laser processing using an Nd:YVO4 laser source. The morphological and compositional characterization of the samples surface was performed by SEM - EDAX. The influence of morphological and chemical surface features on viability, proliferation, adhesion and morphology of human MSC, primary GEpi and GF was investigated by quantitative colorimetric viability assay, SEM and immunofluorescence techniques.

Results: Laser-induced periodic surface structures, exibiting periodicities of several hundreds of nanometers, were found by SEM. The surface roughness slightly increased with increasing the applied laser fluence. Quantitative EDAX analyses showed a linear increase of oxygen concentration with increasing the laser fluence, while the alloying elements content was relatively constant. Cell proliferation assay performed at different time points for cellular models specific for implant abutment zone showed that all laser-textured surfaces induced an increase in viability and proliferation irrespective of cell line, compared to unprocessed Ti-based surfaces. Actin and vinculin cytoskeleton proteins detected by fluorescence microscopy revealed a dynamic cell morphology in response to surface modification. Thus, the laser textured materials allowed for a better attachment, higher cell density and spreading specific for all cell lines tested. In addition, the surface modification induced a modulation of the expression of alpha isoform of actin filaments (a-SMA) marker of fibrosis for GF cells.

Conclusions: The laser nanotexturing of Ti based surfaces for implant abutment promoted cell viability and proliferation, allowed an increased adhesion of human normal primary cells, similar to a normal oral tissue cell phenotype. These results suggest that laser-induced periodic surface structures on titanium-based abutments may confer both soft and hard tissue benefits aiming for improved performance.

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p21-activated kinases as new potential co-targets of tissue transglutaminase for the treatment of ovarian cancer

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Introduction: Ovarian cancer (OC), the 8th most common cancer in women [1], occurs when cells on thesurface of the ovary transform and generate tumors. These cells can detach from the primary tumor, float into the peritoneal cavity and attach to nearby tissues, predominantly to those rich in fats (*e.g.* omentum). Previously, tissue transglutaminase (TG2) was found to be overexpressed in ovarian tumors and involved in multiple stages of cancer progression [2]. It interacts with fibronectin (FN) and promotesintraperitoneal dissemination, which makes it a promising target in treating OC. For this, we developed MT4 - a small molecule inhibitor of the TG2-FN protein-protein interaction [3]. Although effective in preventing OC cell adhesion, MT4 is not effective on all treated cells. In order to identify potential escape pathways, we analyzed the adaptive signaling of OC cells treated with MT4 during attachment onto FN by using SILAC-based phosphoproteomics.

Materials and methods: SILAC labeled OC cells were used in a 30 minutes adhesion assay onto 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. We tested the cytotoxicity of various p21-activated kinase (PAK) inhibitors on OC cells with MTS assay. For the combination of PAK inhibitors and MT4 synergy was evaluated with CompuSyn analysis after a MTS assay. The effect on cell adhesion was evaluated with a 30-minute early attachment assay in which cells treated with PAK inhibitors, MT4 or their combination were seeded onto FN. The fraction of attached cells was quantified. Same experiment was performed for a western blot analysis of the phosphorylated PAKs expression. We also evaluated the effect of drug combinations on cell cycle using flow cytometry.

Results: Phosphoproteomic analysis revealed one class of upregulated proteins (PAKs) that could be promising co-targets of TG2 in treating OC. We tested the combination of MT4 and PAK inhibitors and found synergy only between MT4 and PF-3758309. Also, IPA3-MT4 combination inhibited OC cellsadhesion more than each treatment performed separately. Treatment with MT4 or IPA3 resulted in G2/M phase accumulation, while their combination resulted in S phase accumulation.

Conclusions: Our data provide supportive evidence for promising molecular co-target counteracting escape from TG2-FN interaction inhibition. Further research is required to understand the cross-talk between the two pathways and test the new combination of inhibitors for efficacy in preventing OC dissemination.

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Cytokines and Vitamin D: Key Biomarkers in Heart Failure Therapeutic Strategies

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Introduction: Inflammation is increasingly recognized as a critical factor in the development and progression of heart failure (HF), where it not only contributes to myocardial damage but also exacerbates systemic complications. Among the key inflammatory markers, tumor necrosis factor-alpha (TNF-α) and interleukin-10 (IL-10) play significant and contrasting roles in the pathophysiology of HF. TNF-α is a pro-inflammatory cytokine that promotes myocardial remodeling, apoptosis, and fibrosis, injuring cardiac function and contributing to disease progression. Conversely, IL-10 is an anti-inflammatory cytokine that counteracts the effects of TNF-α by inhibiting its synthesis and mitigating inflammation. IL-10's protective role includes reducing inflammatory responses and preventing further myocardial injury, which can be crucial in slowing the progression of HF. The balance between these cytokines, particularly the TNF-α/IL-10 ratio, has been proposed as a predictive marker for clinical outcomes in HF patients. Additionally, vitamin D, known for its anti-inflammatory and immunomodulatory properties, has been implicated in the modulation of these inflammatory processes. Vitamin D deficiency, common in HF patients, is associated with higher TNF-α levels and lower IL-10 levels, contributing to a pro-inflammatory state that worsens HF outcomes. Therefore, monitoring and modulating vitamin D levels, along with assessing TNF-α and IL-10, could provide valuable insights into the inflammatory status of HF patients and help guide therapeutic strategies aimed at improving prognosis. A prospective clinical study was conducted to investigate the roles of inflammatory markers and vitamin D levels in the management of severe heart failure.

Materials and methods: The study included both male and female patients, with an average age of 65 years, who were undergoing cardiac resynchronization therapy. Biochemical markers were assessed from peripheral blood samples collected at the time of cardiac resynchronization therapy and six months post-intervention. Additionally, 25(OH)-vitamin D and inflammatory cytokine were assessed from blood collected from the coronary sinus during surgery.

Results: The inflammatory cytokine profile demonstrated significant changes, indicating a reduction in systemic inflammation following the partial recovery of cardiac function six months after cardiac resynchronization therapy therapy. Notably, plasma 25(OH)-vitamin D levels, which were initially low in heart failure patients, showed a substantial increase post-intervention in peripheral blood.

Conclusions: Our data provides a clear reflection of the patient's clinical status and offers predictive value for treatment outcomes. These results suggest that incorporating these biomarkers into clinical practice could enhance the management and therapeutic strategies for heart failure.

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Prostacyclin receptor inhibition of TRPM8 function requires involvement of Gq proteins

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Introduction: Prostaglandin I2 (PGI), or prostacyclin, is one of the prostanoids mediating inflammation and pain through activation of the G protein coupled receptor, the prostacyclin receptor (IP-R) [1]. The Transient receptor potential cation channel subfamily M member 8 (TRPM8) exibits strong anti-inflammatory effects upon activation by cold stimuli or chemical agonist such as icilin or menthol [2]. Taken together the opposing functions of both TRPM8 and IP receptors in the regulation of inflammatory pain and their co-expression in certain tissues, we performed an analysis to explore the functional interaction between human TRPM8 and IP receptors.

Materials and methods: We performed intracellular calcium and cAMP determination using bioluminescence genetic probes on HEK293T heterologously expressing both receptors. In order to further validate our data, we performed fluorimetric calcium determinations on a culture of dorsal root ganglion neurons (DRG), which expresses endogenously both TRPM8 and IPR.

Results: TRPM8 induced activation was dose dependently and non-competitively inhibited by the prestimulation of IPR with prostacyclin analogs, effect mediated by the coupling of IPR to Gq/11, not the canonical and well described Gs coupling. We observed the same effects in DRG neurons isolated from mice.

Conclusions: These findings contribute to a better understanding of the mechanisms mediated by TRPM8 and IPR that mediates pain and inflammation.

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Elastin-like polypeptides as nanoparticle platforms for antigen delivery

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Introduction: Elastin-like polypeptides (ELP) are proteins whose primary sequence is inspired by tropoelastin motifs consisting of $(VPGXG)_n$ aminoacid sequence repeats. Due to their aminoacid distribution, these proteins exhibit thermoresponsiveness in form of a reversible phase transition at near physiological temperatures. While soluble and monomeric at room temperature, upon reaching transition temperature, these proteins coalesce into nanometric aggregates which makes them suitable for a plethora of applications. Our study aims to use ELPs to develop optimized antigen delivery systems that may increase vaccine efficiency. To this end, here we describe the search for optimum workflow and parameters for synthesis and analysis of two ELPs and ELP-derived fusion proteins.

Materials and methods: Two pET-25b(+) vectors, each harbouring $(VPGVG)_{96}$ – V96 and $(VPGSG)_{48}(VPGIG)_{48}$ – S48I48 coding sequences, were used to generate fusion proteins with enhanced green fluorescent protein (EGFP) and influenza A H1N1 hemagglutinin ectodomain (HA-ECTO). Briefly, coding sequences for EGFP and HA-ECTO were PCR amplified to generate NdeI recognition sites at each end. A NdeI situs placed at 5′-end of ELPs allowed inserting of either EGFP or HA-ECTO modified sequences. Cloning resulted in multiple positive bacterial colonies that, besides regular PCR colony testing, needed proper orientation check with restriction enzymes. All plasmids were used for transformation in E. coli BL21(DE3) strain. A single colony resulted from each plasmid transformation was used for bacterial cell culture and subsequent IPTG-induced protein expression. Soluble ELP or ELP fusion proteins were collected via cell lysis or cold osmotic shock periplasm extraction. Purification was performed via inverse transition cycling (ITC). Purified proteins were analysed for particle size with dynamic light scattering (DLS) and for phase transition properties using differential scanning calorimetry (DSC) and differential scanning fluorimetry (DSF).

Results: Due to the highly repetitive sequence of ELPs restriction cloning using a NdeI recognition situs was considered most adequate. Vectors with correct sequence and orientation were successfully isolated. Protein expression was achieved for each tested protein with sufficient yield after purification via ITC. DSC and DSF data were generally correlated, showing reversible transition for purified proteins and transition temperatures in agreement with the scientific literature.

Conclusions: Our work aimed at achieving synthesis, purification and physico-chemical data on a set of ELPs and ELPs fusion proteins. Our proof-of concept study established the tuning parameters and optimized workflow for expression and purification of these vehicle proteins. Moreover, the HA-ECTO-ELP fusion proteins obtained here, will be used as models for antigen delivery in future studies concerning their biological activity and feasibility for use in vaccine development.

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How does the bacterial YidC insertase binds mRNA?

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Enhanced antitumor activity of anthocyanins from red grapes and red cabbage after gastrointestinal digestion: effects on colorectal cancer cells

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Introduction: Colorectal cancer (CRC) ranks among the highest in both incidence and mortality of all cancer types. Dietary factors are known to impact CRC development, with anthocyanins—secondary metabolites in fruits and vegetables—demonstrating chemopreventive and antitumoral effects. However, their structure and properties are altered during gastrointestinal metabolization. Therefore, this research focuses on assessing the antitumor effects of anthocyanins from red grapes (*Vitis vinifera*) and red cabbage (*Brassica oleracea* var. *rubra*) on colorectal cancer cells, comparing their efficacy before and after a simulated *in vitro* static digestion.

Materials and methods: Polyphenol extracts from red grapes and red cabbage were obtained by classical maceration in acidified methanol. Starting from these preparations, anthocyanins were further purified using Sep Pack C18 columns. The anthocyanins extracts underwent an *in vitro* static digestion simulation following the INFOGEST protocol, mimicking oral, gastric and intestinal digestion steps. The biochemical profile of both undigested and digested anthocyanin extracts was assessed using HPLC-MS. The antitumor effects of all extracts on DLD-1 and Caco-2 colorectal cell lines was evaluated using the MTT assay. Cell cycle progression and apoptosis were measured by flow cytometry, whereas the cells' confined migration capacity was evaluated using 3D microfluidic devices in DLD-1 cells treated with the digested extracts.

Results: While in the red grapes extract a high diversity on anthocyanins was found, with delphinidin, cyanidin, malvidin, peonidin and petunidin derivates all being present, the red cabbage extract only contained cyanidin derivatives. Following *in vitro* static digestion, the concentration of these compounds decreased, but significant quantities of their metabolites were detected. All extracts possessed strong antitumor activity on both DLD-1 and Caco-2 CRC cell lines in a dose-dependent manner. The undigested extract from grapes yielded better results compared to the cabbage extract, with IC_{50} values of 31,61 µg/mL on DLD-1 cells and 24,73 µg/mL on Caco-2 cell line. Following *in vitro* digestion, the extracts showed significantly increased antitumor activities, with grape extract becoming 1.4-1.9X and cabbage extract growing to be 3-5X more potent against CRC cells. Flow cytometry on DLD-1 cells treated with the digested extracts showed that the mechanisms behind the observed effects involved both apoptosis induction and cell cycle arrest in the S phase. The digested extracts also significantly inhibited confined cell migration of DLD-1 cells in 3D microfluidic devices.

Conclusions: Anthocyanins from food sources have notable *in vitro* antitumor effects, which are further enhanced by gastrointestinal digestion. The anthocyanins metabolites induce apoptosis, inhibit proliferation and hamper cell motility in CRC cells. Therefore, anthocyanins might be promising candidates for CRC prevention and treatment.

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Synthesis and characterization of Buforin derivatives

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Introduction: Antimicrobial peptides are short molecules renowned for their roles in combating pathogens and efficiently penetrating cell membranes [1]. Buforin IIb, a potent antimicrobial peptide consisting of 21 amino acids, demonstrates both antimicrobial activity and effective cell-penetrating abilities [2]. Buforin IIb is a promising candidate for biomedical applications, particularly in cancer therapy due to its ability to target and penetrate cancer cells, enhancing treatment precision. Additionally, its antimicrobial properties hold potential for developing more effective treatments for infections, including those resistant to current therapies, addressing a critical need in the field [3].

Materials and methods: Four buforin derivatives were synthesized using solid-phase synthesis on an automated synthesizer. The synthesis involved sequential addition of amino acids to a resin-bound peptide chain. Peptides were cleaved using a cocktail of trifluoroacetic acid, water, and triisopropylsilane in the Razor device to ensure deprotection of the peptide and the complete removal from the resin. Then, the derivatives were purified by high-performance liquid chromatography (HPLC) to isolate the desired products. The purity and identity of the peptides were confirmed using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS).

Results: The synthesis of buforin derivatives was achieved using solid-phase synthesis, yielding peptides with the expected sequences. The cleavage process using TFA, water, and TIS in the Razor device was efficient, resulting in complete deprotection and cleavage of peptides from the resin. HPLC purification resulted in high-purity peptides, confirmed by distinct chromatographic peaks. MALDI-TOF-MS analysis verified the molecular weights of the purified derivatives, confirming successful synthesis. The buforin derivatives are intended to be tested as antimicrobials.

Conclusions: The successful synthesis and purification of buforin IIb derivatives resulted in peptides with the desired purity and structural integrity, making them suitable for further investigation. These derivatives show promise for addressing antibiotic resistance and enhancing the precision of cancer treatments due to their inherent antimicrobial and cell-penetrating properties.

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A Bioinformatics Approach to Limiting Central Nervous System Inflammation by Targeting TRPV

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Introduction: TRPV1 is a ligand gated non-selective cation channel in the vanilloid receptor family (Caterina et al, 2000), that is found in the membrane of primary afferent neurons in dorsal root ganglia, as well as in central nervous system (CNS) neurons located in the periaqueductal grey, substantia nigra, locus coeruleus and hypothalamus (McGaraughty et al. 2003; Mezey et al. 2000; Toth et al. 2005). In CNS inflammatory processes, TRPV1 overexpression leads to an increased calcium influx that causes damage to the mitochondria and results in cytochrome c release, caspase-3 cleavage (Jihong Xing, 2007). Glutamine supplementation reduces expression of TRPV1 in inflammatory response of macrophages (Lina Duo, 2020), in adipocytes, and influences the release of skeletal muscle cell cytokines (Yixiao Xu, 2022). In this study we put forward the hypothesis that glutamine can be used as a modulator of TRPV1 in CNS neurons that regulate inflammation, nociception, and autonomic response.

Materials and methods: For this, we analysed the interaction between TRPV1 and glutamine using molecular docking methods. Docking was performed using Autodock 4.0 based on the cryo-EM 3D structure of TRPV1 (Protein Data Bank code: 8gfa). The 3D structure of glutamine was extracted from DrugBank.

Results: The analysis found that glutamine binds to the receptor, therefore it has affinity for TRPV1, being a possible modulator of the channel in CNS neurons.

Conclusions: TRPV1 targeted by glutamine has the potential to reduce CNS inflammation, improve pain and autonomic responses, and therefore reduce systemic inflammation overall.

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Inherited Metabolic Diseases: Mitochondrial Involvement in an ATP Synthase Defect as a Deficiency of Assembly Factor, Comparison with a Defect of Urea Cycle Disorder, implications for clinical approach

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Inherited metabolic diseases (IMDs) encompass a diverse array of genetic disorders that disrupt metabolic pathways, often leading to severe clinical manifestations. We focus on the mitochondrial involvement in ATP synthase defect, particularly TMEM70 deficiency diagnosed in a newborn, and compares it with ornithine transcarbamylase (OTC) deficiency, a urea cycle disorder. TMEM70 deficiency results in impaired assembly of the ATP synthase complex, leading to mitochondrial dysfunction characterized by lactic acidosis, hypotonia and hypertrophic cardiomyopathy. The accumulation of metabolic byproducts due to ATP synthesis impairment can trigger secondary complications, including hyperammonemia, which exacerbates mitochondrial stress. Genetic analysis confirmed the presence of the c.317-2A>G mutation in the TMEM70 gene, highlighting the critical role of assembly factors in mitochondrial integrity. In contrast, OTC deficiency directly impairs the urea cycle, leading to elevated ammonia levels that induce mitochondrial dysfunction. Both conditions illustrate how disturbances in mitochondrial metabolism have cascading effects on overall metabolic health. The clinical implications of these findings underscore the necessity for comprehensive diagnostic approaches that include metabolic profiling and genetic testing. Management strategies should address the primary metabolic defect, but also consider the secondary effects. Early intervention and tailored therapeutic approaches, including dietary modifications and potential gene therapy, are essential for improving outcomes in patients with these IMD. This comparative analysis emphasizes the interconnectedness of metabolic pathways and the importance of a clinical approach in managing IMDs.

Keywords: Inherited metabolic diseases, mitochondrial defects, TMEM70, urea cycle diseases, OTC, lactic acidosis, hyperammonemia, next-generation sequencing.