ICGEB International Centre for Genetic Engineering and Biotechnology | Developing Knowledge

ICGEB Research Grants Programme

RESEARCH GRANTS COMPLETED IN 2017

CRP - ICGEB RESEARCH GRANTS COMPLETED IN 2017

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HUNGARY	Lóránt SZÉKVÖLGYI	Structural examination of histone mutations driving human disease
MEXICO	Alejandra OCHOA-ZARZOSA	Innate immune-modulatory properties of plant antimicrobial peptides on mammalian cells during host-pathogen interactions
MOROCCO	Driss IRAQI	A transgenic approach to improve drought and salinity tolerance in wheat
PAKISTAN	Mazhar IQBAL	Structural and antigenic analyses of lipopolysaccharides of typhoidal pathogens
RUSSIA	Nikolay V. GNUCHEV	Role of PGRP-S protein and its complexes with other proteins in anticancer immunodefence
SERBIA	Jelena BEGOVIC	Lactic acid bacteria bioactive compounds as inducers of autophagy
SLOVAKIA	Dusan DOBROTA	Molecular profiling of cancer: biomarkers of progression, therapeutics and predisposition
SLOVENIA	Martina BERGANT MARUSIC	Understanding the molecular basis of infection by oncogenic human papillomaviruses
SOUTH AFRICA	Don A. COWAN	Unravelling the sorghum root microbiome: a route to enhancing crop performance

VENEZUELA	Francisco Alexis RODRIGUEZ-ACOSTA	Molecular cloning and characterisation of metalloproteinases and disintegrins, with therapeutic potential, encoded by cDNAs from venom glands of Venezuelan *Bothrops colombiensis* snake
VIET NAM	Quoc NGUYEN BAO	Host induced gene silencing in the rice blast fungus *Magnaporthe oryzae*

ARGENTINA

Title: Recombinant chimeric MICA: A novel approach to reinstate tumour immunity

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ICGEB Contract No.: CRP/13/002
ICGEB Reference No.: CRP/ARG13-01

Abstract: Most immunotherapies against tumour cells are aimed at restoring anti-tumour immunity. However, they must overcome tumour immune escape mechanisms to efficiently promote eradication of established tumours and metastases. Natural killer (NK) cells and cytotoxic CD8+ T lymphocytes (CTLs) are major players of anti-tumour immunity. Both cells express NKG2D, a major activating receptor involved in tumour cell recognition and destruction. In humans, NKG2D recognises ligands expressed almost exclusively on tumour cells such as the MHC class I chain-related protein A and others. However, tumours subvert the biological function of NKG2D promoting as the proteolytic shedding of MICA, and soluble MICA (sMICA) down-modulation and degradation of NKG2D in NK cells and CTLs. As some cancer patients treated with a blocking anti-cytotoxic T lymphocyte-associated antigen 4 (CTLA4) monoclonal antibody (mAb) develop anti-MICA Ab which correlates with a clinical benefit, our hypothesis was that active induction of anti-MICA Abs in tumour-bearing hosts might be beneficial as they promote clearance of sMICA and restoration of normal expression of MICA on tumour cells, restoration of NKG2D expression on NK cells and CTLs, antibody-dependent cell-mediated cytotoxicity (ADCC)-mediated elimination of tumour cells upon binding to MICA expressed on tumour cells and promotion of tumour-specific CTLs. This project addressed a novel biotechnological approach that might be useful for the treatment of tumour-bearing patients, as monotherapy, as combined therapy or as complementary therapy.

Objectives:

Taking advantage of an established platform suitable to produce highly immunogenic chimeric proteins based on the use of the enzyme lumazine synthase from *Brucella spp.* (BLS), the objective of this project was to produce a highly immunogenic chimeric BLS-MICA protein, use it to induce anti-MICA Abs in tumour-bearing hosts, investigate if these Abs enhance anti-tumour immunity, and elucidate the underlying mechanisms using preclinical models.

Results Obtained:

We have produced the chimeric BLS-MICA protein, and analysed its stability. The protein was purified and used to immunise naïve mice in order to study its immunogenicity. We determined that it was possible to induce high titers of anti-MICA antibodies even without the use of adjuvants. In parallel, we produced mouse cell lines stably expressing MICA (in C57BL/6 background) using a lentiviral expression system. The sera from these animals not only recognised MICA by ELISA (using plates coated with recombinant MICA) but also recognised cell lines that naturally express MICA on their cell surface, as assessed by flow cytometry, indicating that the immunisation with BLS-MICA elicits antibodies that recognise the native MICA protein. Next, using a therapeutic approach of passive immunisation of mice that carry palpable tumours, we demonstrated that anti-MICA antibodies promoted tumour regression, which in some cases led to animals that remained tumour-free for up to two months. In addition, using a prophylactic approach, we demonstrated that active immunisation of mice with BLS-MICA (and the subsequent induction of anti-MICA antibodies) induced a slower/delayed tumour growth when these mice were challenged with MICA-expressing tumours but not when they were challenged with control tumours. We explored the underlying immunological mechanisms responsible for the delayed tumour growth and observed that animals treated with anti-MICA antibodies display an increase in intratumoural pro-inflammatory macrophages and antigen-experienced CD8 T cells, suggesting that these antibodies reinstate an efficient anti-tumour immunity.

Results Unforeseen in the Original Project:

As part of the original project, we planned to analyse the serum concentration of soluble MICA (sMICA) using a commercial ELISA kit. However, as such ELISAs failed systematically, we started to develop an in-house system to assess sMICA concentration by an alternative method that we are optimising. Preliminary data indicate that it is possible to detect sMICA with an acceptable detection limit. Thus, from an initial drawback, we found a new opportunity to explore and develop a novel and alternative system to detect sMICA. This unforeseen aspect of the project may lead to a new product and to file a new patent.

Publications:

Torres, N., Regge, M.V., Secchiari, F., Friedrich, A.D., Spallanzani, R.G., Raffo Iraolagoitia, X.L., Núñez, S.Y., Sierra, J.M., Ziblat, A., Santilli, M.C., Gilio, N., Almada, E., Lauche, C., Pardo, R., Domaica, C.I., Fuertes, M.B., Madauss, K.P., Hance, K.W., Gloger, I.S.,

Zylberman, V., Goldbaum, F.A., Zwirner, N.W. Restoration of antitumor immunity through anti-MICA antibodies elicited with a chimeric protein. 2020. J. Immunother. Cancer **8(1)**, e000233.

BOSNIA AND HERZEGOVINA

Title: Genetic and bioactivity characterisation of some endemic B&H Lamiaceae
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ICGEB Contract No.: CRP/14/004
ICGEB Reference No.: CRP/BOS14-02
Abstract: Lamiaceae species, widely used in culinary and folk medicine, are well known for their antioxidant, anti-inflammatory, anti-microbial and anti-cancer activity. This research aimed to determine potential antimicrobial, geno/cytotoxic, cytostatic and apoptotic activity of endemic B&H Lamiaceae: *Acinos orontius*, *Satureja horvatii*, *S. subspicata*, *Thymus bracteosus* and *Micromeria pulegium*. Also, selected nrDNA, chloroplast and AFLP markers were used to examine genetic diversity. *In vitro* studies of examined plant extracts, regardless the solvent use, showed no antimicrobial activity nor genotoxic, cytotoxic or cytostatic effects. Following *in vivo* studies confirmed absence of genotoxicity of *Satureja* species aqueous extracts in Wistar mice. Analysis of relative expression of apoptosis associated genes suggests insignificant, between species more or less pronounced upregulation of pro-apoptotic genes. Some extracts exhibit upregulation of anti-apoptotic genes. Obtained results do not oppose to the widen use of these plants. Molecular characterisation showed great diversity among selected species. Genetic diversity was the lowest within *Micromeria pulegium* and the highest within *Satureja subspicata* and *Thymus bracteosus*. *Satureja subspicata* samples subgroup corresponding to the sampling locality and indicate a possible geographically linked genetic differentiation.
Objectives:
The main objective of our project was analysis of potential biological activity of five endemic Lamiaceae species [*Micromeria pulegium* (Rochel) Benth, *Acinos orontius* (K.Maly) Šilić, *Satureja horvatii* Šilić, *S. subspicata* Bartl. ex Vis and *Thymus bracteosus* Vis ex Bentham] present in B&H flora. Within the biological activity we investigated antimicrobial, genotoxic, cytotoxic, cytostatic and apoptotic activity of aqueous and DMSO extracts of five selected plant species. Molecular characterisation was performed as well, utilising specific markers: analysis of chloroplast DNA performed by PCR-RFLP method, AFLP method for quick overview of whole genome and sequencing of DNA region within nuclear ribosomal DNA (nrDNA).
Results Obtained:
Extracts of five endemic B&H Lamiaceae showed no antimicrobial activity against all tested bacterial and fungal strains. Conducted analysis did not reveal significant genotoxic or cytotoxic activity of tested plant extracts, regardless solvents used. Also, dose dependent trends in frequencies of observed parameters were not registered. As it was expected, positive controls were significantly different from all other treatments, both for genotoxicity as well as cytotoxicity parameters. For extracts of two *Satureja* species *in vivo* testing of genotoxicity on mice reticulocytes was performed. Results indicate antigenotoxic activity of *Satureja* extracts due to statistically significant decrease in frequency of micronuclei in polychromatic erythrocytes/reticulocytes in mice treated with the highest concentration of *Satureja* aqueous extracts. Evaluated apoptotic activity of *Satureja* extracts strongly suggest that the highest concentration has anti-apoptotic activity, unlike the lowest concentration where the upregulation of several pro apoptotic genes has been registered. Aqueous extracts of *A. orontius*, in two lower concentrations, showed downregulation of pro-apoptotic genes in BCL-2 family (BOK1 and BIK), as well as upregulation of anti-apoptotic gene BCL2A1. Highest concentration also showed up-regulation of BNIP3L (NIX) and BID genes (pro-apoptotic, members of BCL-2 family). All samples showed overexpression of *GZMB* gene, which is crucial for target cell apoptosis. Downregulation od pro-apoptopic genes was observed in DMSO extracts as well, with only significant up-regulation of CFLAR gene. Extracts of *Thymus bracteosus* had the least impact on relative expression of apoptosis – related genes than any other extract. Human blood cultures treated with *M. pulegium* extracts, regardless of solvent or concentration applied showed uniform upregulation of pro-apoptotic genes, which can suggest that *M. pulegium* extracts have pro-apoptotic influence on normal human leucocytes in culture.
Genetic diversity of the five studied Lamiaceae species was analysed based on cpDNA (PCR-RFLP trnL intron and *mat*K sequencing) and nuclear DNA markers (AFLP with three primer-pair combinations and ITS1 and ITS2 sequencing). Five partial *mat*K and five concatenated nrDNA consensus sequences have been deposited in the GenBank database. Also, *mat*K sequences from all savoury species were submitted and mined in BOLD Systems v3 Database. Differential polymorphisms (SNPs) within partial *mat*K sequence (420 bp) were identified for four species with *Satureja subspicata* and *S. horvatii* exhibiting identical sequence. However, ITS2 sequences provided better resolution for molecular-genetic differentiation analysis among the analysed Lamiaceae species. AFLP analysis showed comparable genetic diversity parameters for four species

with *Micromeria pulegium* exhibiting lower degree of heterogeneity. *S. subspicata* samples from subgroups that correspond to the locations of sampling, which indicates possible link between genetic differentiation and geographical distance. With the exception of *S. subspicata* and *S. horvatii* where certain degree of genetic mixture is evident, three species are clearly genetically differentiated.

Extracts of selected Lamiaceae do not exhibit harmful bioactive effects that would oppose their traditional use. However, due to the endemicity of this species and the fact that they do not exhibit notable beneficial effects, their use should be restricted and controlled, as they are easily replaced with widespread savoury species. Considering molecular characterisation, no added value from phytopharmaceutical exploitation of the five studied Lamiaceae species can be predicted. In light of their limited distribution and established genetic diversity parameters, their contribution to biodiversity of the region surpasses their ethno botanical potential. Therefore, the five studied Lamiaceae species should be allowed to continue unhindered.

BRAZIL

Title: Development of genetically modified bacteria for heavy metal removal from industrials wastewaters

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ICGEB Reference No.: CRP/BRA13-03

Abstract: We are developing a genetically engineered microorganism that can remove heavy metals from water, wastewater, sediments and soil, with the final goal to be used in industrial plants, wastewater treatments and bioremediation of contaminated areas. The project involves the insertion of modified metallothioneins (MTs) genes from the tropical oyster *Crassostrea rhizophorae* in the heavy metal resistant bacteria *Cupriavidus metallidurans*. MTs have the high cysteine content, strategically positioned to make connections with tetrahedral metal atoms, ensure greater affinity to these elements than any other substance. Their binding energy (MT-metal cluster - 2000 $kJ.mol^{-1}$) is up to 1000 times higher than that of physical absorption (20 $kJ.mol^{-1}$) or chemosorption (500 $kJ.mol^{-1}$). The laboratory experiments show that *Escherichia coli* with the modified *Saccharomyces cerevisiae*'s MT increases the efficiency of removal of cadmium in solution in 15 to 20 times compared to bacteria that lack this modification. We have genetically manipulated the sequence of the MT gene to create 8 variants (wild type + seven combinations of α and β domains in tandem) of the protein, addressed to the outer bacterial membrane. These proteins are now subject of biophysical studies to examine their properties (stability and energy of liaison) and microbial studies to determine their heavy metal removal potential.

Objectives:

The project done with the help of ICGEB is the part of the bigger project, which has milestones, and their respective status at the time of submitting the proposal was:

(i) Cloning and sequencing of the metallothionein of the oyster *Crassostrea rhizophorae* (done);
(ii) Construction of the tandem repeats of the MT domains in one artificial gene (done);
(iii) Plasmid construction with fusion protein addressed to the bacterial membrane (done);
(iv) Transformation of *Cupriavidus metallidurans* CH34 bacteria with the plasmid (done - partially);
(v) Test of metals adsorption in the laboratory using the newly built bacterial strain;
(vi) Biophysical characterisation of the fusion protein (in progress);
(vii) Ultra structural characterisation of the newly built bacterial strain (next steps);
(viii) Bacterial conjugation and segregational stability of the engineered strain (next steps);
(ix) Scaling the newly built bacterial strain production in bioreactors in the laboratory (next steps);
(x) Test of heavy metal removal from industrial wastewaters in bioreactors in the laboratory (next steps);

Objectives planned to be achieved during the ICGEB financing help are given as the milestones (v) and (vi).

Results Obtained:

Test of metals adsorption in the laboratory using the newly built bacterial strain: During the project, our focus was to: determine the optimal Cd ion concentration for induction of newly built strain; compare the adsorption capacity of the wild bacterial strain (*C. metallidurans* LMG1195), newly built strain (*C. metallidurans* MTαβ4), and newly built strain pre-induced with Cd ion (pre-induced *C. metallidurans* MTαβ4); check if it is possible to use bacterial biomass after an overnight rest. The optimal concentration of Cd ion for induction is 0.3 mM Cd ion solution. The biomass can be used over the overnight rest at the room temperature, without losing its bioaccumulation ability and capacity. The research was conducted with the 1 mM metal solution, even though the literature shows that concentration present in waste waters can be much less compared to this one. With the pre-induced *C. metallidurans* MTαβ4, we managed to remove up to 1563 mg of Cd ions/kg of bacterial biomass, and 687.77 mg of Ni ions /kg of bacterial biomass. The better adsorption capacity was present with the higher biomass concentration (14.19 g/L) and the shorter time of exposure (6 h for Cd ions and 3 h for Ni ions). However, the results show that bacteria, with its newly built accumulation mechanism, have the potential for bioaccumulation. The future experiments will be dedicated to optimisation of bacterial concentration needed for each type of solution, as well as what is needed time of exposure.

Biophysical characterisation of the protein: Due to the course of our research, it appeared that would be useful to know the Ni release potential, so the biophysical characterisation of the protein went in that direction. For this purpose, MTαβ4 was cloned into the pGEX-6P-1 expression vector and the recombinant fusion protein MTαβ4-GST was heterologous expressed in *E. coli* Bl21. MTαβ4-GST was purified by affinity chromatography using Glutathione-Sepharose4B and MTαβ4 was recovered by enzymatic cleavage. Nickel release from the purified MTαβ4 was tested and confirmed

by measuring its transfer from Ni7-MT to PAR induced by the presence of the H_2O_2/H_2O redox couple.

Results Unforeseen in the Original Project:

The funds of ICGEB provided us with enough manpower to conduct additional experiments: Screening metal adsorption capacity (Co, Zn, and Cu ions) for pre-induced *C. metallidurans* MTαβ4; Screening metal adsorption capacity (mixture of Cd, Co, Ni, Fe, and Cu ions) for pre-induced *C. metallidurans* MTαβ4; Screening metal adsorption capacity for rare elements (1mM La ion solution) for pre-induced *C. metallidurans* MTαβ4; Recovery of metal ions from the biomass by using HCl, EDTA, and CO_2.

The newly built bacterial strain was able to bio-accumulate ions of Co, Zn and Cu from the solutions of initial concentration 1mM. Moreover, the highest bioaccumulation for Zn ions was after 0.5 h (7540 mg/kg of bacterial biomass), while accumulation of Co and Cu ions was lower, with 288 mg/kg of bacterial biomass after 3 h and 971 mg/kg of bacterial biomass after 0.5 h, respectively. The synthetic mixture of metal ions was based on literature data about the metal solution in mining industry (Cd 4.7 mg/L, Co 80.46 mg/L, Cu 110.5 mg/L, Fe 10.89 mg/L, Ni 110.2 g/L). The bioaccumulation of Ni ions was the highest, during the whole monitored time (12 h), with accumulated ions 16 g/kg of bacterial biomass after 12 h. For the rest of ions the highest accumulation was after one hour (Co 7.7 mg/kg, Cu 1594 mg/kg, and Fe 2174 mg/kg), after which started the ion release. The Cd ions had the constant ions decrease in biomass. However, it should be stressed that the strain was pre-induced with Cd ions, and probably their release allows the bioaccumulation of other ions. Later on, during the experiments with La, the best result was after 6 h of exposure, 14410 mg/kg of bacterial biomass. The screening possibility of metal ions recovery from the biomass was also done, with a test of 3 different desorbents. HCl showed as the most effective desorbent, even though the most destructive one.

All the gotten data helped us to submit a request for a patent on bacteria and its design.

The part of milestones, planned for the later part of the project, was also done during this funding - scale up of bacteria growth in 100 L fermenter. This was the proof of the concept with the wild strain, and resulted in yield of 32.42 g/L dry weight.

This result, together with the above-mentioned results for newly built bacteria strain, gives the foundation for the further development of bacteria and its process of bioaccumulation. The research might result in efficient product for the wastewater treatment, and other problems present in modern industries regarding the heavy metals.

CHILE

Title: Role of $Sox2^+$ ependymal cells in spinal cord regeneration in *Xenopus*

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Abstract: Unlike mammals, the African clawed frog, *Xenopus laevis*, can regenerate the spinal cord before metamorphosis (R-stages), a capability that is lost after metamorphosis climax (NR-stage). This feature makes *Xenopus* a unique experimental paradigm to identify the cellular and genetic mechanisms involved in spinal cord regeneration that are no longer present in non-regenerative organism such as mammals. Studying this basic biological question should provide new insights to understand why regeneration fails in mammals and how to design novel therapeutic approaches to improve recovery in human patients.
In this project we propose that "$Sox2^+$ ependymal cells activated in regenerative stages are required for spinal cord regeneration in *Xenopus* and regulate this process through two mechanisms: providing a permissive substrate for axonal regeneration and producing new neurons by neurogenesis".

Objectives:
(i) To characterise the $Sox2^+$ ependymal cells and its niche in regenerative and non-regenerative stages;
(ii) To determine if $Sox2^+$ ependymal cells are necessary and sufficient for spinal cord regeneration;
(iii) To establish the mechanisms used by $Sox2^+$ ependymal cells to promote spinal cord regeneration.

Results Obtained:
We have demonstrated that in regenerative animals a massive and rapid proliferation of $Sox2/3^+$ cells occurs in the spinal cord in response to injury and that this proliferation is necessary for reconstruction of the spinal cord and functional recovery. Contrary to that, no effect such as this is observed in non-regenerative animals suggesting that activation of Sox2/3 cells is a key mechanism to allow spinal cord regeneration. In addition we have performed a detail characterisation of the ventricular zone of regenerative and non-regenerative spinal cords using electron microscopy and immunofluorescence that depicts the profound difference in the cellular composition of both stages.
To address the possible role of Sox2/3 cells in promoting axon regeneration we have set-up spinal cord transplantation experiments and found that cells from regenerative spinal cord promote axon growth and axon regeneration in non-regenerative animals.

Publications:
Muñoz, R., Edwards-Faret, G., Moreno, M., Zuñiga, N., Cline, H., Larraín, J. Regeneration of *Xenopus laevis* spinal cord requires Sox2/3 expressing cells. 2015. Dev. Biol. **408**, 229-243
Lee-Liu, D., Méndez-Olivos, E.E., Muñoz, R., Larraín, J. The African Clawed frog *Xenops laevis*: a model organism to study regeneration of the central nervous system. 2016. Neurosci. Lett. (in press)
Edwards-Faret, G., Muñoz, R., Méndez-Olivos, E., Lee-Liu, D., Tapia, V.S. ,Larraín, J. Spinal cord regeneration in *Xenopus laevis*. *2017.* Nature Protocols **12**, 372-389

CHINA

Title: Osteoporosis therapy by silencing bone formation inhibitor

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ICGEB Contract No.: CRP/13/005
ICGEB Reference No.: CRP/CHN13-02

Abstract: Currently, major concerns about the safety and efficacy of RNA interference (RNAi)-based bone anabolic strategies still exist because of the lack of direct osteoblast-specific delivery systems for osteogenic siRNAs. Here we screened the aptamer CH6 by cell-SELEX, specifically targeting both rat and human osteoblasts, and then we developed CH6 aptamer–functionalised lipid nanoparticles (LNPs) encapsulating osteogenic pleckstrin homology domain-containing family O member 1 (Plekho1, also known as CKIP-1) siRNA (CH6-LNPs-siRNA). Our results showed that CH6 facilitated in vitro osteoblast-selective uptake of Plekho1 siRNA, mainly via macropinocytosis, and boosted in vivo osteoblast-specific Plekho1 gene silencing, which promoted bone formation, improved bone microarchitecture, increased bone mass and enhanced mechanical properties in both osteopenic and healthy rodents. These results indicate that osteoblast-specific aptamer-functionalised LNPs could act as a new RNAi-based bone anabolic strategy, advancing the targeted delivery selectivity of osteogenic siRNAs from the tissue level to the cellular level.

The HECT-type ubiquitin ligase Smurf1 (Smad ubiquitination regulatory factor-1) plays the prominent role in regulation of bone formation, embryonic development, and tumorigenesis by directing the ubiquitin-proteasomal degradation of specific targets. In contrast with RING-type E3s, the catalytic HECT domain of Smurf1 firstly binds to and then transfers ubiquitin (Ub) molecules onto the substrates. The Smurf1-Ub interaction is required for Smurf1 catalytic ligase activity to promote substrate degradation. However, so far specific regulators or compounds controlling Smurf1-Ub interaction and the ligase activity have not been identified. Here we report two small molecule compounds targeting Ub binding region of HECT domain interrupt Smurf1-Ub contact, inhibit Smurf1 ligase activity and stabilise BMP signal components Smad1/5 protein level. Furthermore, these compounds increase BMP signal responsiveness and enhance osteoblastic activity in cultured cells. These findings provide a novel strategy through targeting Smurf1 ligase activity to potentially treat bone disorders such as osteoporosis.

Objectives:

(i) To examine the cell-selective distribution of CKIP-1 siRNA;
(ii) To test the cell-specific CKIP-1 knockdown efficiency;
(iii) To determine the dose-response pattern and persistence of gene silencing;
(iv) To determine bone histomorphometric parameters, bone mass and mechanical properties;
(v) To detect the accumulation of CKIP-1 siRNA in hepatocyte and liver-related toxicity;
(vi) To design small molecular inhibitors of CKIP-1 or Smurf1.

Results Obtained:

Results from immunohistochemistry and flow cytometry showed that CH6 achieved *in vivo* osteoblast-specific delivery of CKIP-1 siRNA and no obvious accumulation of siRNA in hepatocytes and PBMCs (objective 1.1) and boosted in vivo osteoblast-specific gene silencing (objective 1.2). In addition, dose-response experiment showed that CH6-LNPs-siRNA achieved almost 80% gene knockdown efficiency at the siRNA dose of 1.0 mg/kg and subsequently maintained 12 days for over 50% gene silencing (objective 1.3). MicroCT re-construction, bone histomorphometric analysis and mechanical testing further confirmed that CH6 facilitated bone formation, leading to improved bone micro-architecture, increased bone mass and enhanced mechanical properties in ovariectomised rodents with established osteopenia (objective 1.4). Furthermore, CH6-LNPs-siRNA achieved much better bone anabolic action when compared to our previously developed $(DSS)_6$-liposome-siRNA. No obvious toxicity was observed in rats after single/multiple dose(s) of CH6-LNPs-siRNA (objective 1.5). All these results indicated that osteoblast-specific aptamer-functionalised LNPs could act as a novel RNAi-based bone anabolic strategy and advance selectivity of targeted delivery for osteogenic siRNAs from tissue level toward cellular level.

BMP signaling play significant role in osteoblastic bone formation. The ubiquitin ligase Smad ubiquitination regulatory factor-1 (Smurf1) negatively regulates bone morphogenetic protein (BMP) pathway by ubiquitinating certain signal components for degradation. Thus, it can be an eligible pharmacological target for increasing BMP signal responsiveness. On one hand, we established a strategy to discover small molecule compounds that block the WW1 domain of Smurf1 from interacting with Smad1/5 by structure based virtual screening. Our selected hits could reserve the protein level of Smad1/5 from degradation by interrupting Smurf1-Smad1/5 interaction and inhibiting Smurf1 mediated ubiquitination of Smad1/5. Further, these compounds increased BMP-2 signal responsiveness and the expression of certain downstream genes, enhanced the osteoblastic activity of myoblasts and osteoblasts. On the other hand, the catalytic HECT domain of Smurf1

firstly binds to and then transfers ubiquitin (Ub) molecules onto the substrates. The Smurf1-Ub interaction is required for Smurf1 catalytic ligase activity to promote substrate degradation. Here we report two small molecule compounds targeting Ub binding region of HECT domain interrupt Smurf1-Ub contact, inhibit Smurf1 ligase activity and stabilise BMP signal components Smad1/5 protein level. Furthermore, these compounds increase BMP signal responsiveness and enhance osteoblastic activity in cultured cells. Our work indicates targeting Smurf1 for inhibition could be an accessible strategy to discover BMP-sensitisers that might be applied in future clinical treatments of bone disorders such as osteoporosis (objective 1.6).

Publications:

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Liang, C., Guo, B., Wu, H., Shao, N., Li, D., Liu, J., Dang, L., Wang, C., Li, H., Li, S., Lau, W.K., Cao, Y., Yang, Z., Lu, C., He, X., Au, D.W., Pan, X., Zhang, B.T., Lu, C., Zhang, H., Yue, K., Qian, A., Shang, P., Xu, J., Xiao, L., Bian, Z., Tan, W., Liang, Z., He, F., Zhang, L., Lu, A., Zhang, G. Aptamer-functionalised lipid nanoparticles targeting osteoblasts as a novel RNA interference-based bone anabolic strategy. 2015. Nat. Med. **21(3)**, 288-294

Zhang, Y., Wang, C., Cao, Y., Gu, Y., Zhang, L. Selective compounds enhance osteoblastic activity by targeting HECT domain of ubiquitin ligase Smurf1. 2016. Oncotarget (in press)

EGYPT

Title: Surface immobilisation of saponin hydrolase enzyme on agricultural residues: Novel biocatalysts for soybean saponin hydrolisis

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ICGEB Contract No.: CRP/14/009

ICGEB Reference No.: CRP/EGY14-04

Abstract: Soyasapogenol B (SB) is known to have many biological activities such as, hepatoprotactive, anti-inflammatory, anti-mutagenic, antiviral and anticancer activities. Enzymatic conversion of soybean saponin (SS) to SB was carried out using saponin hydrolase (SH) extracted from *Aspergillus flavus,* which was screened and selected on the basis of its ability to produce high SH activity of 64.26 U/ml. This strain was capable of expressing three SH forms: extracellular, intracellular and cell wall bound forms. SH cell bound enzyme contributed more than 75% of the total enzymatic activity in the production medium. The conditions for maximum production and extraction of SH from *A. flavus* were investigated. Taguchi design model predicted that maximum cell bound SH activity of 81.73 U/g would occur when the medium contained glucose and SS at concentrations of 2 and 1.3%, adjusted at pH 9 and incubated for 4days. The highest SH extraction yield of 85.83% was achieved when 0.25 M Tris–HCl lysis buffer supplemented with 1% Triton X-100 for 24h at 4-25^{o}C and pH 8.0 was applied. SH partial purification was achieved by applying successively acetone precipitation, lypholisation, dialysis and anion exchange chromatography on Fractogel EMD DEAE-650 (S) to the extract. The partially purified enzyme was immobilised on different supports by physical adsorption, covalent binding or entrapment. Natural cellulosic supports (rice straw, bagasse, sawdust, loafa, and corncob), chitosan, sodium alginate and Eupergit C were used for SH immobilisation. Covalent immobilisation of the enzyme was carried out on different supports functionalised by i) glutaraldehyde (GA), ii) N'-diisopropylcarbodiimide (DIC) and N-hydroxysuccinimide (NHS), iii) sodium periodate (PI) or iv) polyethyleneimine (PEI) and GA. Among different employed immobilisation procedures and supports, covalent binding to Eupergit C and bagasse activated by DIC and NHS were proved to be the best two supports which recovered 46.5 and 37.1% of the original added enzyme activity, respectively. SH immobilisation conditions on both Eupergit C and bagasse functionalised by DIC and NHS including; DIC and NHS concentrations, immobilisation time, pH, temperature, SH initial concentration, support weight were determined. Under optimised immobilisation conditions, immobilised SH on Eupergit C and functionalised bagasse recovered 87.7 and 83.3% of its original activity, respectively. Compared to free SH, immobilised SH on Eupergit C and functionalised bagasse showed higher optimum pH, activation energy, half-lives, lower deactivation constant rate. Also, SB productivity produced by immobilised SH on Eupergit C and functionalised bagasse after 24h incubation were higher by 2.4 and 2.2 fold higher compared to free SH (87.7 and 83.3 vs. 37.5% respectively) under identical conditions.

Objectives:

(i) Screening of different fungal isolated strains for producing soybean saponin hydrolase (SH) that can degrade soybean saponin and generate soyasapogenol B (SB) and selection of the most potent one;
(ii) Optimisation of different parameters for enhanced production of saponin hydrolase;
(iii) Partial purification of SH by different methods;
(iv) Screening and chemical functionalisation of different support materials including cellulosic agricultural leftovers such as rice straw, sugarcane bagasse, or sawdust, etc. as solid matrix for enzyme immobilisation;
(v) Optimisation of the protocols for covalent binding and cross-linking immobilisation methods, with regard to parameters such as temperature, pH, concentration, stirring rate, additives, and cross-linking agent;
(vi) Comparison between free and immobilised enzymes concerning the ability to produce soyasapogenol B from soybean saponin and their thermal stability.

Results Obtained:

Out of twenty-two isolated strains, *Aspergillus flavus* was proved to be the best SH producer. So, it was selected on the basis of its ability to hydrolyse SS and produce the highest yield of SB. SH production by *A. flavus* has been modeled and optimised using Taguchi's orthogonal methodology. The optimum conditions for maximum production of SH activity and SB yield were; 1% glucose, 1.3% SS, medium pH 7 and 4 days incubation period. An enhancement of 137.3 % in SH production compared to the original production medium was detected and SB yield was significantly improved after application of Taguchi method. *Aspergillus flavus* was capable of expressing three SH forms; extracellular, intracellular and cell-wall bound forms. SH cell bound enzyme constituted to more than 75% of the total enzymatic activity in the production medium.

The sequential extraction process of SH cell bound enzyme revealed that 47.5% of SH was cytosolic and the rest (52.5%) was associated with the cell wall. The highest SH extraction yield (85.83%) was achieved when 0.25 M Tris–HCl lysis buffer supplemented with 1% Triton X-100 for 24h at 4-25^{o}C and pH 8.0 was applied. Under these optimised conditions, *A. flavus* SH yield increased from 23.6 to 85.83%. SH partial purification was achieved by applying successively acetone precipitation, lypholisation, dialysis and anion exchange chromatography on Fractogel EMD DEAE-650 (S) to the extract. The final enzyme preparation was 7. 3 fold purer than the crude extract. The partially purified enzyme was immobilised on different supports by physical adsorption, covalent binding or entrapment. Covalent binding to Eupergit C and bagasse activated by N'-diisopropylcarbodiimide (DIC) and N-hydroxysuccinimide (NHS) were proved to be the best two supports which recovered 46.5 and 37.1% of the original added enzyme activity, respectively. Under optimised immobilisation conditions, immobilised SH on Eupergit C and activated bagasse recovered 87.7 and 83.3% of its original activity, respectively. Compared to free SH, immobilised SH on Eupergit C and activated bagasse showed higher optimum pH, activation energy, half-lives, lower deactivation constant rate. Also, SB productivity produced by immobilised SH on Eupergit C and activated bagasse after 24h incubation were 2.3- and 2.2-fold higher compared to free SH (87.7 and 83.3 vs. 37.5%, respectively) under identical conditions.

Results Unforeseen in the Original Project:

(i) *A. flavus* SH enzyme was mainly cell-wall bound enzyme and consequently needed to be extracted from fungal cell wall. Optimisation of its extraction condition was also investigated;
(ii) Investigation of SH immobilisation on synthetic supports; e.g Eupergit C.

Publications:

Amin, H.A., Mohamed, S.S., Abo Elsoud, M.M., Awad, H.M. Statistical optimisation of *Aspergillus flavus* cell bound saponin hydrolase production by Taguchi DOE methodology. 2016. Der Pharmacia Lettre **8(19)**, 108-114

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Amin, H.A., Secoundo, F., Amer, H., Helmy, W.A., Ahmed, F.M. Improvement of *Aspergillus flavus* saponin hydrolase thermal stability and productivity via multipoint covalent immobilisation. 2017. Journal of Molecular Catalysis B: Enzymatic (in press)

FYR MACEDONIA

Title: Genetic screening for mutations in GH1 and GHRHR in a cohort of patients with isolated growth hormone deficiency (IGHD); Elucidating the genetic cause(s) for a distinct association of IGHD and myopathy.

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ICGEB Reference No.: CRP/MAC13-01

Abstract: Among the cohort of children with GHD in the project we found a *LHX4* mutation in a 14 years old boy with short stature. At the age of 5 years his gait was unstable. A progressive myopathy ensued. Tests of pituitary reserve showed partial IGHD (8.2 ng/ml). Muscle biopsy showed congenital myopathy of undefined aetiology. Targeted re-sequencing with a panel containing probe sets for enrichment and analysis of >4,800 clinically relevant genes, targeting 12Mb of the human genome revealed the c.250C>T (R84C) *LHX4* mutation. His father is healthy, with no myopathy or pituitary deficiencies, but has the same *LHX4* mutation. The Charcot Marie Tooth gene mutation was also noted, but did not correspond with the clinical, biochemical and pathological findings.

In another patient with GHD we found clinical signs of Fanconi anaemia, which we further proved by genetic analysis. Fanconi anaemia (FA) is a rare autosomal recessive or X-linked disorder characterised by clinical and genetic heterogeneity. Typical clinical features include developmental abnormalities in major organ systems, endocrine abnormalities, bone marrow failure and a high predisposition to cancer development. We present two new FA patients, a 2-year old girl from Macedonia compound heterozygote for the known c.190–256_283+1680del2040dupC and a novel c.3446_3449dupCCCT mutation. This patient had tests of pituitary reserve in a GHD range. A 10-year-old girl from Kosovo homozygote for the novel c.3446_3449dupCCCT mutation in FANCA gene.

We also found an 11 old patient with hypoplasia of the right kidney and hypospadias was found to be SRY negative, 46, XX. His intelligence was normal (IQ 92). The behaviour, growth and development were all normal. ACTH test was normal. SNP array for copy number variations (CNV's) showed a unique 550 kb duplication involving *SOX3, RP1-177G6,* and *CDR1* genes, and the microRNA MIR320D2. This CNV was absent in 13,839 controls. A SRY negative 46,XX male with renal hypodysplasia was found to have an exceedingly rare duplication involving the *SOX-3* gene, proving its role in sex determination and suggesting its evolvement in kidney development.

In the same cohort of the SGA children, an eight-year old child presented with painful non-traumatic swelling of both forearms. Subsequently, additional lesions formed at the knees (at age of 10 years), lower back (at age 14 years) and the back of the neck (at age 15 years). Cervical spine showed ossification of the ligamenta flava. At 16 years of age, whole exome sequencing identified an *ACVR1* mutation (c.983G>A; p.G328E), that had previously been identified in patients with FOP. This is in contrast to previous reports that described other patients with this rare variant with a later onset and milder phenotype.

Rubinstein–Taybi syndrome (RSTS) is an autosomal dominant congenital disorder characterised by broad thumbs and halluces, growth retardation, facial dysmorphisms, skeletal abnormalities and mental retardation. We report a girl in whom only mild skeletal abnormalities were observed. Her stature is short (-2.6 SD). Tests of pituitary growth hormone (GH) reserve showed normal GH levels. Targeted re-sequencing was performed with probe sets for enrichment and analysis of the coding regions of >4,800 clinically relevant genes and revealed no *CREBBR* or *EP300* genes.

A 3-year old boy was found to have short stature and GHD. The same result was found in his mother, who also had hypoplasia of the fourth metatarsal bones. There were no *CREBBR* or *EP300* gene alterations, while molecular karyotyping revealed a del8p.

We investigated 100 children born SGA for associated congenital anomalies of kidney and urinary tract and obesity. We revealed 4 obese and grown up children with BMI above 98th percentile. Two children had hyper-insulinemia and insulin resistance and underwent treatment with metformin. Seven children were diagnosed with CAKUT and had very low birth parameters.

We revealed 7 (7.0%) SGA born children with congenital anomalies of the urinary tract. Their mean birth weight was very low 1855 gr (-3.93 SDS) and the birth length 45.57cm (-2.17 SDS), as well. A significant growth failure with reduced weight and BMI were noticed at the time of diagnosis. Three children revealed with unilateral kidney agenesis, 2 had hypo-dysplastic kidneys and in 2 children was found vesicoureteral reflux.

Brothers DJ and MJ (males, affected) with both unaffected parents. The family quartet has Turkish background. The clinical features include hypopituitarism, myopathy, ataxia, facial dysmorphisms,

accumulated trunk fat, small hands and feet. In the meantime the younger brother developed chronic glomerulonephritis gradually leading to renal failure. There was a single gene with compound heterozygous variants passing the segregation filter where each parent is heterozygous for one of the variants (RNPC3:NM_017619:exon6:c.613C>T:p.R205X and RNPC3:NM_017619: exon13:c.1420C>A:p.P474T).
Those results are further under investigation. Cricks institute in London created a murine knock out model, while the Great Ormond Street Hospital is investigating fibroblasts from the patients with various functional analysis.
We further examined *IGF1R* (Insulin-like Growth Factor 1 Receptor) gene in patients with Small for Gestational Age (SGA).
Small for gestational age (SGA)-born children are a heterogeneous group with few genetic causes reported. We analysed 89 children born SGA who stayed short (-3.2 +/-0.9 SD) within the first 5 years of age. Another 43 children with similar characteristics were analysed for the presence of deletions/duplications of sequences within the following three genes: IGF1R, located on chromosome 15q26, IGFBP3, located on 7p13 and IGFALS, located on 16p13.3. The presence of IGF1R gene alterations was done Multiplex Ligation Probe Amplification (MLPA) and/or by screening the PCR products of all coding IGF1R exons by dHPLC followed by direct sequencing of conspicuous fragments.
The first cohort revealed a novel heterozygous, synonymous variant c.3453C>T and a second novel heterozygous 3 bp deletion (c.3234_3236delCAT) resulting in one amino acid deletion (p.Ile1078del). Interestingly, the first patient had normal serum levels of IGF1. Surprisingly, the second patient had unusually low IGF1 serum concentrations (-1.5 SD), which contrasts previously published data where IGF1 levels rarely are found below the age adjusted mean. The second cohort of patients did not detect any gene alterations.
IGF1R gene alterations were present in 2 of 89 short SGA children. It is of note that the patients did not have any dysmorphic features (small head size, micrognatia), nor developmental delay.

Objectives:

(i) To determine the frequency of GH1 gene deletions (6.7 kb, 7.6 kb,7 kb) and Growth hormone releasing hormone receptor GHRHR(E72X) gene mutations in patients with IGHD;
(ii) To identify the genotype/phenotype correlations in patients with IGHD;
(iii) To elucidate the genetic cause(s) of the peculiar association between hypopituitarism and myopathy in the four described patients. The characteristic phenotypes of the patients, the diverse ethnic background and the extensive testing so far suggest that the use of WES will result in the identification of novel genetic defects.

Results Obtained:

(i) We characterised a child with LHX4 mutation and hypopituitarism with myopathy.
(ii) We also characterised two brothers with myopathy and hypopituitarism in whom there was a single gene with compound heterozygous variants passing the segregation filter where each parent is heterozygous for one of the variants (RNPC3:NM_017619:exon6:c.613C>T:p.R205X and RNPC3:NM_017619: exon13:c.1420C>A:p.P474T). A murine model and functional analysis are currently underway at Cricks institute and GOSH in London, UK.
(iii) We further examined *IGF1R* (Insulin-like Growth Factor 1 Receptor) gene in patients with Small for Gestational Age (SGA). Small for gestational age (SGA)-born children are a heterogeneous group with few genetic causes reported. IGF1R gene alterations were present in 2 of 89 short SGA children. We found a novel heterozygous, synonymous variant c.3453C>T and a second novel heterozygous 3 bp deletion (c.3234_3236delCAT) resulting in one amino acid deletion (p.Ile1078del). Interestingly, the first patient had normal serum levels of IGF1. Surprisingly, the second patient had unusually low IGF1 serum concentrations (-1.5 SD), which contrasts previously published data where IGF1 levels rarely are found below the age adjusted mean. It is of note, that the patients did not have any dysmorphic features (small head size, micrognatia), nor developmental delay.
(iv) In another patient with GHD we found clinical signs of Fanconi anaemia, which we further proved by genetic analysis. Fanconi anaemia (FA) is a rare autosomal recessive or X-linked disorder characterised by clinical and genetic heterogeneity. Typical clinical features include developmental abnormalities in major organ systems, endocrine abnormalities, bone marrow failure and a high predisposition to cancer development. A 2-year old girl was compound heterozygote for the known c.190–256_283+1680del2040dupC and a novel c.3446_3449dupCCCT mutation. This patient had tests of pituitary reserve in a GHD range. A 10-year old girl from Kosovo homozygote for the novel c.3446_3449dupCCCT mutation in FANCA gene.
(v) We also found an 11 old patient with hypoplasia of the right kidney and hypospadias was found to be SRY negative, 46, XX. His intelligence was normal (IQ 92). The behavior, growth and development were all normal. ACTH test was normal. SNP array for copy number variations (CNV's) showed a unique 550 kb duplication involving *SOX3, RP1-177G6,* and *CDR1* genes, and the microRNA MIR320D2. This CNV was absent in 13,839 controls. A SRY negative 46,XX male with renal hypodysplasia was found to have an exceedingly rare duplication involving the *SOX-3* gene, proving its role in sex determination and suggesting its evolvement in kidney development.

(vi) In the same cohort of the SGA children, an eight-year old child presented with painful non-traumatic swelling of both forearms. Subsequently, additional lesions formed at the knees (at age of 10 years), lower back (at age 14 years) and the back of the neck (at age 15 years). Cervical spine showed ossification of the ligamenta flava. At 16 years of age, whole exome sequencing identified an *ACVR1* mutation (c.983G>A; p.G328E), that had previously been identified in patients with FOP. The phenotype is in contrast to previous reports that described other patients with this rare variant with a later onset and milder phenotype.
(vii) Rubinstein–Taybi syndrome (RSTS) is an autosomal dominant congenital disorder characterised by broad thumbs and halluces, growth retardation, facial dysmorphisms, skeletal abnormalities and mental retardation. We report a girl in whom only mild skeletal abnormalities were observed. Her stature is short (-2.6 SD). Tests of pituitary growth hormone (GH) reserve showed normal GH levels. Targeted re-sequencing was performed with probe sets for enrichment and analysis of the coding regions of >4,800 clinically relevant genes and revealed no *CREBBR* or *EP300* genes.
(viii) A 3-year old boy was found to have short stature and GHD. The same result was found in his mother, who also had hypoplasia of the fourth metatarsal bones. There were no *CREBBR* or *EP300* gene alterations, while molecular karyotyping revealed a del8p.
(ix) We investigated 100 children born SGA for associated congenital anomalies of kidney and urinary tract and obesity. We revealed 4 obese and grown up children with BMI above 98th percentile. Two children had hyperinsulinemia and insulin resistance and underwent treatment with metformin. Seven children were diagnosed with CAKUT and had very low birth parameters.

Results Unforeseen in the Original Project:

(i) LHX4 mutation was found to go with the mutation for Charcot Marie Tooth disease. This was not in agreement with the clinical presentation, biochemistry or histopathological findings. And remains to be elucidated.
(ii) In two brothers with myopathy and hypopituitarism there was a single gene with compound heterozygous variants passing the segregation filter where each parent is heterozygous for one of the variants (RNPC3:NM_017619:exon6:c.613C>T:p.R205X and RNPC3:NM_017619: exon13:c.1420C>A:p.P474T). Molecular mechanisms are unknown and expected to be elucidated from the murine model and the functional analyses on patient's skin fibroblasts.
(iii) There was an increased number of children with CAKUT and SGA.
(iv) A 2-year old girl was compound heterozygote for the known c.190–256_283+1680del2040dupC and a novel c.3446_3449dupCCCT mutation was found to have GHD and Fanconi anaemia.
(v) We found a novel heterozygous, synonymous variant c.3453C>T and a second novel heterozygous 3 bp deletion (c.3234_3236delCAT) resulting in one amino acid deletion (p.Ile1078del) in two SGA children. Unforeseen, the first patient had normal serum levels of IGF1, while, the second patient had unusually low IGF1 serum concentrations (-1.5 SD), which contrasts previously published data. The patients did not have any dysmorphic features (small head size, micrognatia), nor developmental delay.
(vi) Rubinstein–Taybi syndrome (RSTS) is an autosomal dominant congenital disorder was found in two children.
(vii) Whole exome sequencing identified an *ACVR1* mutation (c.983G>A; p.G328E), that had previously been identified in patients with FOP. The phenotype is in contrast to previous reports that described other patients with this rare variant with a later onset and milder phenotype.

Publications:

Gucev, Z., Tasic, V., Plaseska-Karanfilska, D., Konstantinova, M.K., Stamatova, A., Dimishkovska, M., Laban, N., Polenakovic, M. LHX4 gene alterations: Patient report and review of the literature. 2016. Pediatr. Endocrinol. Rev. **13(4)**, 749-755

HUNGARY

Title: Structural examination of histone mutations driving human disease

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ICGEB Reference No.: CRP/HUN13-01

Abstract: Nucleosomes are the main building blocks of chromatin organisation controlling a number of biochemical processes from gene expression regulation to DNA replication and repair. Many of these functions are mediated through histone posttranslational modifications (PTMs) present at various amino acid side chains, acting in a combinatorial manner via partially understood mechanisms. Mishaps in the proper writing, reading or erasing of epigenetic marks can induce a drastic change in the underlying chromatin signalling pathways, leading to the formation of pathological diseases. Lysine 27 to methionine substitution of the replication independent histone H3.3 drives the appearance of the aggressive brain tumour glioblastoma multiforme (GBM) in infants. Formation the disease phenotype has not been fully understood at the molecular level and remains an open question.

Objectives:

In the current project, we performed a comprehensive structural examination of the histone H3K27M mutation driving pediatric glioblastoma, starting from nucleosome nanostructures to the *in vivo* kinetic analysis of mutant nucleosomes. We applied various *in vitro* and *in vivo* model systems (*E. coli*, *S. cerevisiae*, and human cell lines) and state of the art genetic and biophysical approaches to reveal how the histone H3 lysine 27 amino acid point mutation could prime disease formation.

Results Obtained:

The structural and the kinetic properties of the H3.3K27M mutant nucleosomes did not reveal a significant difference compared to the wild-type nucleosomes, we found a remarkable shift and transcription-dependent re-distribution of EZH2 (the catalytic subunit of the PRC2 histone H3K27 methylase complex) in live cells that was independent from the K27M mutation.

Results Unforeseen in the Original Project:

The lnRNA-binding of EZH2 prompted us to perform an optimised RNA-DNA hybrid immunoprecipitation sequencing (DRIP-Seq) so that we establish the genome-wide topography of RNA-DNA hybrid binding sites and correlate them with the chromosomal atlas of EZH2 binding sites. The optimised DRIP workflow we developed has become a standalone methodological story.

Publications:

Lóránt, S., Kunihiro, O., Alain, N. Initiation of meiotic homologous recombination: Flexibility, impact of histone modifications and chromatin remodelling. 2015. Cold Spring Harb. Perspect. Biol. **7(5)**, a016527

MEXICO

Title: Innate immune-modulatory properties of plant antimicrobial peptides on mammalian cells during host-pathogen interactions

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ICGEB Contract No.: CRP/13/011
ICGEB Reference No.: CRP/MEX13-01

Abstract: Infectious diseases caused by bacterial pathogens are one of the leading causes of morbidity and mortality worldwide, most of them have evolved to adhere or invade mammalian cells, including epithelial cells that form the natural cellular defense barriers. Intracellular colonisation allows microorganisms to evade the immune system and the antimicrobial treatment, favouring the increase of resistant microbial strains, which constitutes a severe health problem. Therefore, it is necessary to develop new and improved strategies to control bacterial infections. Antimicrobial host defense peptides, commonly named "antimicrobial peptides" (AP) are produced by all complex organisms as well as some microbes which have diverse antimicrobial activities involved in host protection against invading bacteria, viruses and fungi. Host antimicrobial peptides have an important immunomodulatory function besides their antibacterial activity. However, the actions of plant antimicrobial peptides (PAP) on innate immune response (IIR) of mammals are poorly known.

Objectives:

To determine the effect of plant antimicrobial peptides on innate immune response of mammalian cells during host-pathogen interactions.

Results Obtained:

During this research we established that plant defensins could act as immunomodulators on mammalian cells, being g-thionin from *Capsicum chinense* a better immunomodulatory peptide than thionin Thi2.1 (*Arabidopsis thaliana*). These effects result in a better defense of bovine mammary epithelial cells against *Staphylococcus aureus.*

Results Unforeseen in the Original Project:

Plant defensins, such as g-thionin from *Capsicum chinense,* surprisingly can induce the activation of transcriptional factors related to inflammation and innate immune response in mammals.

Publications:

Díaz-Murillo, V., Medina-Estrada, I., López-Meza, J.E., Ochoa-Zarzosa, A. Defensin gamma-thionin from *Capsicum chinense* has immunomodulatory effects on bovine mammary epithelial cells during *Staphylococcus aureus* internalisation. 2016. Peptides **78**, 109-118

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MOROCCO

Title: A transgenic approach to improve drought and salinity tolerance in wheat
Principal Investigator: Driss Iraqi, Biotechnology Unit, Centre Régional de la Recherche Agronomique de Rabat, Institut National de la Recherche Agronomique (INRA) BP 415, Avenue Ennasr, Rabat, Morocco. Tel: +212-661-164616, 660-157242, Fax: +212-537-775530, E-mail: iraqid@yahoo.fr
ICGEB Contract No.: CRP/13/012
ICGEB Reference No.: CRP/MOR13-02
Abstract: Drought and salinity are the most important environmental stress affecting the wheat production worldwide, causing a severe decrease in productivity. Transfer of resistance to these abiotic stresses, using traditional approaches is limited because of the complexity of the traits. However, genetic transformation can help in improving these traits. In this project, we have used genes involved in drought and salinity tolerance, namely, *Hardy* and *SINA* genes from *Arabidopsis thaliana* for transformation of Moroccan wheat varieties using the construct with *bar* gene as selectable marker. In order to achieve better transformation and regeneration, culture media were optimised for better embryogenic callus induction, genetic transformation and regeneration using mature and immature embryos as explants. The genetic transformation using biolistic approach resulted in transgenic wheat plants selected in medium containing Basta herbicide. Furthermore, leaf painting assays and PCR analysis using primers complementary to *bar* gene also confirm integration of transgene in wheat plants. Further physiological analysis is needed to study the drought and salinity tolerance in the developed transgenic wheat plants. A total of five students (1 M.Sc. and 4 B.Sc. degree students) completed training/research and four Ph.D. students are continuing their thesis studies.
Objectives:
(i) Transformation of bread wheat and durum wheat with genes known to be involved in drought tolerance;
(ii) Empower young researchers and students to implement genetic engineering in the crop improvement programs.
Results Obtained:
The present study tested and identified the favourable media for induction and regeneration *in vitro* from mature and immature embryos of Moroccan bread and durum wheat varieties. In both species, the regenerative potential is affected by the genotype, media and the nature of explant used. The identified media were successfully used for genetic transformation and regeneration of Moroccan bread wheat and durum wheat. The genetic transformation with Arabidopsis *Hardy* and *SINA* genes resulted in regeneration of transgenic plantlets in bread wheat and durum wheat respectively. Further studies will be conducted for phenotyping and molecular analysis of the obtained transgenic plants.
A total of five students (1 M.Sc. and 4 B.Sc. degree students) completed training/research and four Ph.D. students are continuing their thesis studies.
Publications:
Aadel, H., Ahansal, K., Udupa, S.M., Gaboun, F., Abdelwahd, R., Douira, A., Iraqi, D. Effect of genotypes and culture media on embryogenic callus induction and plantlet regeneration from mature and immature bread wheat embryos. 2016. Rom. Agric. Res. **33**, 61-69
Aadel, H., Abdelwahd, R., Gaboun,F., Udupa, S.M., Diria, G., El mouhtadi, A., Ahansal, K., Douira, A., Iraqi D. *Agrobacterium*-mediated genetic transformation of bread wheat (*Triticum aestivum L.*) genotypes using mature embryos tissues. 2017. Cereal Research Communications (in press)

PAKISTAN

Title: Structural and antigenic analyses of lipopolysaccharides of typhoidal pathogens

Principal Investigator: Mazhar Iqbal, National Institute for Biotechnology and Genetic Engineering (NIBGE), P.O. Box 577, Jhang Road, Faisalabad, Pakistan. Tel: +92-41-2651475-79, 299, Fax: +92-41-2651472, E-mail: hamzamgondal@yahoo.com, hamzamgondal@gmail.com, mazhar@nibge.org

ICGEB Contract No.: CRP/13/014
ICGEB Reference No.: CRP/PAK13-03

Abstract: Typhoid is a major public health problem worldwide; in particular it occurs endemically throughout the developing world. This disease is caused by *Salmonella enterica* subspecies enterica serovar Typhi (*S. Typhi*). It annually affects about 33 million people worldwide and is associated with about 600,000 deaths. Unfortunately, typhoid is known to be the 4th largest cause of death in Pakistan, there is no national vaccination program being offered yet. Technically, this is mainly because of the unavailability of any fully effective vaccine in the world, which can cover all types of typhoidal ailments in all age groups.

Traditionally, Vi (capsular) polysaccharide of *S. Typhi* is being imported and used as a vaccine. Besides being a poor quality immunogen for children of less than five years of age and need repeated doses, this is also not effective against *Salmonella Paratyphi* A and Vi negative strains of *S. Typhi*. The Vi-conjugate vaccine developed by NIH is also not effective against Vi-negative *S. Typhi* and *S. Paratyphi* A. Moreover, the Vi based vaccines are exerting the potential selective pressure or niche in the favour of Vi-negative *S. Typhi* and *S. Paratyphi* A.

To address this problem a conjugate vaccine effective against all prevalent typhoidal pathogens is required. In fact, the success of effective conjugate vaccine development will heavily rely on the structural characteri sation of lipopolysaccharides (LPS) of *S. Typhi* strains, which are the key component of the conjugate vaccine. LPS are vital to both the structural and functional integrity of the Gram-negative bacterial outer membrane.

LPS confer stability to the bacterial membrane and protect from bacteriophages, certain antibiotics as well as the host defense mechanism during infection. Initially, it was proposed that Vi-polysaccharide is the main virulent in *S. Typhi*. However, the isolation of Vi-negative *S. Typhi* from typhoid patient highlighted the complexity existing in the pathogenicity of *S. Typhi*, which rendered the need to understand the complete surface chemistry of these pathogens.

In this study, the unambiguous analysis of LPS of Vi-positive and Vi-negative strains of *S. Typhi* has been conducted. After characteri sation of these bacterial strains on molecular basis, they were grown in 20-litre fermenter to obtain sufficient cell biomass. Subsequently, LPS were isolated through hot phenol method. The purified LPS were subjected to mild acid hydrolysis in order to fractionate the lipid A and O-specific polysaccharide components.

Lipid A samples from *S. Typhi* Vi-positive and Vi-negative were characteri sed by GC-MS (fatty acid profiling) and ESI-MS/MS analysis. Both of these strains demonstrated mixture of highly heterogeneous lipid A structures with significance difference to each other in fatty acid acylations, phosphorylations and covalent modifications. *S. Typhi* Vi-positive sample indicated the higher abundance of mono-phosphorylated tetra and pentaacylated lipid A structures. Whereas, *S. Typhi* Vi-negative sample represented the higher intensities of mono and bisphosphorylated hexa and heptaacylated lipid A variants.

Both of the lipid A samples significantly differ with respect to covalent modification, i.e., Vi-negative lipid A demonstrated elevated level of the addition of L-Ara4N, while Vi-positive sample showed higher activities of PagL. LpxO activity (addition of 2-OH) is more pronounced in higher acylated (hexa & hepta) structures in Vi-negative sample, while this phenomenon is reversed in pentaacylated variant in Vi-positive lipid A sample. Presence of few unusual lipid A structures such as the occurrence of C10 and C14 secondary fatty acyl chains at 2' position of the distal glucosamine have been observed in both samples.

It can be concluded that S. Typhi Vi-negative isolate, being without Vi-polysaccharide or biofilm around it, needs more investment on its LPS and more importantly on lipid A to make its membrane more compact and resistant as compared to Vi-positive isolate. Having higher amount of mono and bisphosphorylated hexa and heptaacylated lipid A structures, Vi-negative isolate can anchor higher number of OSP repeating unit, which can give additional protection to this bacteria. However, since the hexa and heptaacylated variants over activate host immune response and can generate hot spots of endotoxicity, therefore, these structures have been covalently modified effectively with the addition of L-Ara4N, which can give additional protection to the bacteria against the host antimicrobial cationic peptides. Moreover, these modifications also help the bacteria to modulate or evade the host immune response, which can favor bacteria during its persistent infection. Considering all the aforementioned complexities, *S. Typhi* Vi-negative strain seems to evolve into an advance stage of pathogenicity with respect to endotoxicity, as compared to Vi-positive strain.

Complete analysis of O-specific polysaccharide (OSP) is very complicated and generally required more than one technique to elucidate the types of sugar, their linkages and substitutions. Currently, OSP samples of both strains were chemically characteri sed by GC-MS and NMR techniques. Both of these techniques necessarily complimented each other. The results generated from these techniques, illustrated that OSP of two isolates of *S. Typhi* (Vi-positive and Vi-negative) comprised of different O-specific polysaccharide differing not only in the nature of sugar residues present in repeating units but also their positions. OSP sample of Vi-positive strain constituted -[→3)-α-D-Manp-(1→4)-α-L-Rhap-(1→3)-[α-D-Glcp-(1→4)]α-D-Galp-(1→]- as repeating unit, while Vi-negative strain represented a significantly different repeating unit [→2)-[α-Tyvp-(1→3)]α-D-Manp-(1→4)-α-L-Rhap-(1→3)-α-D-Galp-(1→]-.

Although the carbohydrate backbones of the OSPs of both the strains have same sugars but they differed on their linkage positions. In Vi-positive strain, glucose present at branched position is linked with galactose via a 1-4 linkage while in Vi-negative strain instead of glucose tyvelose is linked to mannose sugar via 1-3 linkage. Tyvelose is more hydrophobic in nature than glucose and its presence on branched position provide a hydrophobic coating to the bacterial cell surface of Vi-negative strain of *S. Typhi*. Its presence may provide additional protection to the bacterial cell from the host defense mechanism, especially in the absence of Vi-capsule. LPS as well as OSP of both the strains showed in-vitro antigenicity in double-immunodiffusion assay.

In short, lipid A and OSP components of LPS from *S. Typhi* Vi-positive and Vi-negative strains have been structurally and functionally characteri sed. These information, no doubt will be helpful in designing multivalent potent conjugate vaccine against typhoidal pathogens in future.

RUSSIAN FEDERATION

Title: Study of the role of PGLYRP1 centered protein complexes in human immune defense
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Abstract: We have recently discovered that protein PGRP-S is involved in several different processes, that allow human lymphocytes to kill cancer cells. But this protein do not acts alone but always in complex with several different proteins. Depending on the partner in this interaction complex PGRP-S plays different role in anticancer immune-defense. In this project we plan to investigate role of complexes PGRP-S with other proteins in this field, mechanisms that allow PGRP-S to perform different roles and regulation of these processes. This research is vital for anticancer studies.

Objectives:

(i) PGRP-S/Hsp70 complex. Secretion by lymphocytes, regulation of activity. Mechanisms of cancer cell death induced by PGRP-S/Hsp70 complex;
(ii) Role of other PGRP-S complexes (PGRP-S/Mts1, PGRP-S/HspBP1 and others) in anticancer defense;
(iii) Lymphocytes that kill cancer cells. Mechanisms of recognition, killing, regulation of anticancer activity.

Results Obtained:

First year: In the literature there are two different points of view on chemotactic properties of PGLYRP protein. One author claim that this protein, released by human neutrophyles, is chemotactic and attracts leucocytes, another one states that recombinant PGLYRP1 protein do not possess any chemotactic activity. These controversies are resolved only if PGLYRP1 released by human neutrophyles is secreted with some other component that alters its chemotactic properties. Indeed earlier we have found that PGLYRP1 protein in complex with Hsp70 protein gains cytotoxic activity, and both of these complex components are not cytotoxic. We have tested if PGLYRP1 alone is possess chemoattractive activity to human blood cells and do not find it. Next we prepare complexes of some proteins that are known to form a complex with PGLYRP1 protein: Hsp70, HspBP1 and S100A4. Only PGLYRP1-S100A4 complex possess strong chemotactic effect to human leucocytes. From this point we start to investigate properties of this complex. We have found that this complex is formed from 1 molecule of PGLYRP1 and 2 molecules of S100A4, and strongly attracts two subpopulations of human leucocytes: NK cells and T-lymphocytes. Within an hour all the NK cells are moved to the lower chamber of Beuden camera, as well as about 20% of CD8+ and 10% of CD4+ T lymphocytes. Other cells are not affected. This effect starts to develop at nanomolar concentration of PGLYRP1-S100A4 complex in dose dependent manner. For NK the effect starts at 10 times lower concentration of PGLYRP1-S100A4 complex, but the saturation was reached at the same concentration – 10-7M. First part of our work help us to characteri se complex of two recombinant proteins and state that PGLYRP1 protein alone is not chemotactic, but it is a complex of two proteins that possess chemotactic activity. Further on we start to search if this complex is artificial or it is released by some human blood cells. We search different cell subfractions as well as total blood populations for traces of PGLYRP1 and S100A4 proteins and found that this complex of two proteins is secreted by neutrophils and monocytes subpopulations of human blood, as well as by some CD8+ and CD4+ T lymphocytes. Confocal microscopy supports our finding, and we found that these cells have complex of this two proteins inside and are able to release it without need of some stimulation. It is very interesting that no additional stimulation is required to induce this secretion. We speculate that this complex may attract antiviral or anticancer cells to travel with immune response inducing cells to help them effectively do their work.

Second year: Tag7 (also known as peptidoglycan recognition protein PGRP-S, PGLYRP1), an innate immunity protein, interacts with Hsp70 to form a stable Tag7-Hsp70 complex with cytotoxic activity against some tumour cell lines. In this study, we have analy sed the programmed cell death mechanisms that are induced when cells interact with the Tag7–Hsp70 complex, which was previously shown to be released by human lymphocytes and is cytotoxic to cancer cells. We show that this complex induces both apoptotic and necroptotic processes in the cells. Apoptosis follows the classic caspase-8 and caspase-3 activation pathway. Inhibition of apoptosis leads to a switch to the RIP1-dependent necroptosis. Both of these cytotoxic processes are initiated by the involvement of TNFR1, a receptor for TNF-alpha. Our results suggest that the Tag7–Hsp70 complex is a novel ligand for this receptor. One of its components, the innate immunity protein Tag7, can bind to the TNFR1 receptor, thereby inhibiting the cytotoxic actions of the Tag7–Hsp70 complex and TNF-alpha, an acquired immunity cytokine.

Third year: We performed a comparative analysis of accumulation in human peripheral blood mononuclear cells (PBMC) populations of CD3+CD4+ cells under the action of the cytokine IL-2 and innate immune protein Tag7. Both proteins were added to the PBMC and then using the flow cytofluometry we determined the number of CD3+CD4+lymphocytes at different day of incubation. CD3+CD4+ lymphocytes constitute a significant part of PBMC, and with time of incubation, their percent increases, reaching a maximum at 6 days. The total curve and the obtained mean values for the content of CD3+CD4+ cells was not much different when activated by the cytokine IL-2 or by Tag7protein. Then we isolated using magnetic separation the fraction of CD3+CD4+ cells from the total PBMC pool after 6 days of incubation with protein Tag7 and examined the specificity of cytotoxic effect of these cells against target cancer cells. Purified via magnetic separation CD3+CD4+ fraction of lymphocytes was added to different tumour cell lines, and the cytotoxic activity was tested. We use K562, Molt-4, HeLa and L-929 cell lines. CD3+CD4+ lymphocytes activated by Tag7 protein killed only K562 and Molt-4, cells that do not carry HLA-antigen. There was observed no traces of cytotoxic activity against HeLa and L929 cells. The highest cytotoxic activity was shown against K562 cells (about 25% dead cells). Thus, Tag7 activated CD3+CD4+ subpopulation of lymphocytes was able to kill only the HLA-negative tumour cells, as was also shown during the activation of lymphocytes by the cytokine IL-2. Previously, we have found that after 6 days of incubation with IL-2 CD4+CD25+ lymphocyte subpopulation was activated. Therefore, we wondered whether activated by Tag7 protein CD4 T cells are also CD25 positive. Also with the help of magnetic separation were selected in CD3+CD4+CD25+ and CD3+CD4+CD25 - subpopulations after 6 days incubation of PBMC with Tag7. The cytotoxic activity was observed only for CD3+CD4+CD25+ subpopulation, a subpopulation of CD3+CD4+CD25- was unable to cause lysis of K562 cells. Thus, the above results indicate that the cytokine IL-2 and protein of innate immunity Tag7 induce after 6 days incubation, the activation of CD3+CD4+CD25+ lymphocytes. To test, whether FasL and Fas are involved in the cytotoxic action of the Tag7-activated subpopulation, we study the cytotoxic activity using the inhibition by specific antibodies. Preincubation of CD4+CD25+ T lymphocytes with anti-FasL antibody or preincubation of K562 cells with anti-Fas antibody led to complete disappearance of cytotoxic activity, while the incubation of cells in the presence of antibodies to granzyme had no effect on cytotoxicity. The results indicate that, as with the activation of lymphocytes IL-2, CD4+CD25+ T lymphocytes kill their target cells through FasL-Fas interaction. Next, we tested how these lymphocytes recogni se their target. We investigated whether Tag7 is presented on the surface of CD4+CD25+ T lymphocytes, activated and Tag7. About 17% of CD3+CD4+CD25+ cells also bear Tag7. Next, we tested whether lymphocyte Tag7 and Hsp70 on the surface K562 cells are involved in the cytotoxic process. One can see that preincubation of CD4+CD25+ lymphocytes with specific antibodies to Tag7 or preincubation of K562 cells with antibodies to Hsp70 blocked cytotoxic activity, confirming that the binding of lymphocyte's Tag7 with Hsp70 on the surface of tumour cells is necessary for subsequent killing through FasL-Fas interaction. Cytotoxic CD3+CD4+CD25+ lymphocytes, activated under the action of the Tag7 are not Treg cells. From literature data it is known that CD4+CD25+ lymphocytes are often referred to as Treg (T regulatory) subpopulation of cells that in the body are responsible for the inhibition of immune response and survival of tumour cells. It has been shown that Treg cells in addition to CD4 and CD25 antigens do not carry the CD127 antigen, unlike other populations of CD4+ cells. We decided to test whether our cells exhibiting antitumor activity, are Treg cells. To this end, we have isolated CD3+CD4+CD25+, CD3+CD4+CD25+Tag7+ and CD3+CD4+CD25+Tag7- subpopulations using magnetic separation and stained cells with phenotype CD4+CD25high with CD127 antibodies. The results flow cytometry analysis show that CD127 antigen is located on the same cells that have Tag7 on the surface. To confirm these results we isolated CD4+CD25+CD127+ and CD4+CD25+CD127- subpopulations of lymphocytes and tested their cytotoxic activity. A subpopulation of CD4+CD25+CD127+ was active, causing the death of 20% of the tumour cells, while CD4+CD25+CD127- population did not show cytotoxic properties. Thus, we were able to show that under the action of Tag7 in human PMBC on day 6 is induced the subpopulation of CD3+CD4+CD25+CD127+FasL+Tag7+ lymphocytes, which are not Treg cells. These cells are able to lyse tumour cells that have lost major histocompatibility complex, and therefore immune to classic adaptive immune response. Thus CD4+CD25+ subpopulation is also heterogeneous in their functions: the fraction of Treg inhibits the action of lymphocytes activated IL-2 and Tag7. The fraction of CD4+CD25+CD127+ kills tumour cells, immune to the classical adaptive immune response. These results highlight the diversity of functional activities of CD4+ T lymphocytes.

Results Unforeseen in the Original Project:

(i) Tag7-Hsp70 complex interacts with TNFR1 receptor of well-known cytokine TNF-alpha
(ii) Treg cells are not cytotoxic to cancer cells;
(iii) Tag7 alone and Mts1 alone cannot induce chemotaxis of human lymphocytes. Only complex Tag7-Mts1 induces movement of cells.

Publications:

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SERBIA

Title: Lactic acid bacteria bioactive compounds as inducers of autophagy
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Abstract: Autophagy presents an adaptive response to unfavourable conditions and represents a major route for degradation of aggregated cellular proteins and dysfunctional organelles. Autophagy is induced by deprivation of nutrients, hormones, energy and different types of stresses. Recently, lactic acid bacteria (LAB) are being identified as potential inducers and producers of factors involved in the process of autophagy.
Results of screening and testing of 41 different LAB extracts for autophagy-promoting potential revealed that nine spent culture supernatants (SCS) and four exipolysaccharides from different LAB exhibited this potential as measured through LC3II/LC3I ratio after treatment of HL-1 cells with extracts. According to the results of Western blot and qPCR: *Lb. paracasei* BGSJ2-8, *Lb. plantarum* BGKP7, *Lb. paracasei/casei* BGMK2-10a, *Lb. sucicola* BGLMM7 had highest pro-autophagic potential. However, autophagy induction in HL-1 cells was associated with reduction of HL-1 cell viability, suggesting the potential of bacterial extracts to suppress tumour cell growth, hypothetically through induction of autophagic (type II) cell death. In support of this, HL-1 death caused by bacterial SCS correlated with induction of the expression of Beclin1, which was previously shown to mediate autophagic cell death in carcinoma cell lines. Furthermore, bacterial SCS arrested HL-1 cell cycle in S/G2 phase, reduced Cyclin B1, phospho H3 and Cdk1 expression, which are all important markers of cell cycle arrest. This research could be the basis for future research that would examine the potential of lactobacilli in treatment of soft tissue cancers, which are rare, but also of more common epithelial tumours both *in vitro* and *in vivo.*
Objectives:
The idea of this project was to screen bacterial collection from Laboratory for Molecular Microbiology to find strain (strains) of lactic acid bacteria (LAB) with the potential to induce autophagy in rat cardiomyocytes. Experiments performed during the first year of the project aimed at selecting different cell fractions of different LAB species for potential to induce autophagy *in vitro* in cardiomyocytes grown in culture. During the second year a focus was to test all 13 bacterial extracts selected during the first year in dose response experiments. Also the objective was to assess the expression of genes in HL-1 cells relevant for autophagy induction (Becn1, SQSTM, UVRAG and CatD) by using qPCR.
Based on the results of the first two years of the project, the third year of the project was dedicated to confirm the effect of four extracts most potent in inducing autophagy in HL-1 cells in at least four replicates for each extract. The assays included: analysis of LC3 expression using Western blot, qPCR analysis of the expression of genes of interest, transfection assays with HcRed-LC3 and also cell cycle analysis. Additionally, due to newly discovered potential effect of the extracts on cell cycle the plan was to irradiate primary fibroblasts from rats and check for the potential of bacterial extracts to arrest irradiated cells in S and/or G2/M phases of cell cycle.
Results Obtained:
Results of screening of 41 different LAB extracts for autophagy-promoting potential revealed that nine spent culture supernatants (SCS) and four exipolysaccharides from different LAB exhibited this potential as measured through LC3II/LC3I ratio after treatment of HL-1 cells with extracts. Also SCS prepared from *Lactobacillus casei* BGMK3-24 has autophagy supressing activity (1.72-fold). Exopolysaccharides (EPS) are most potent inducers of autophagy whilst S-layer proteins have neither potential autophagy promoting nor autophagy supressing activity. On the basis of the results of second year of the project, we selected four bacterial extracts (SCS) that are most potent in inducing autophagy, according to the results of Western blot and qPCR: *Lb. paracasei* BGSJ2-8, *Lb. plantarum* BGKP7, *Lb. paracasei/casei* BGMK2-10a, *Lb. sucicola* BGLMM7. Autophagy induction by bacterial SCS correlated with reduction of HL-1 cell viability, as assessed by propidium iodide staining and flow cytometry. This suggests that the autophagy induced by bacterial extracts was probably related to autophagic (type II) cell death, which has important implication for cancer treatment, since HL-1 are tumour cells. Our further actions during the project were thus oriented towards assessment of HL-1 cell cycle modulation by bacterial extracts. In parallel, we tested the expression of Beclin1, as important tumour suppressor and autophagic cell death mediator. Additionally, to evaluate the selectivity of bacterial extracts only towards tumour cells, we included non-tumour myoblast C2C12 line in analysis. Since BGLMM7 SCS showed the highest antiproliferative potential, we continued the analysis with this bacterial extract. According to flow cytometric data, BGLMM7 SCS modulated cell cycle in both HL-1 and C2C12 cell lines. Specifically,

cells were arrested in G2 phase of cell cycle. Western blot analysis revealed that the treatment of HL-1 cells with BGLMM7 SCS caused a decrease in CCNB1 expression. However this was not the case with C2C12 cell line. According to the results of immunofluorescence, treatment of HL-1 cells with BGLMM7 SCS caused a decrease of H3 (phospho S10) positive spots in nuclei of both HL-1 and C2C12 cells. Also, decreased nuclear staining of Cdk1 in BGLMM7 SCS treated HL-1 cells was obtained, while there was not change in Cdk1 expression in C2C12 cells.
We observed redistribution of Becn1 from nucleus to cytoplasm in BGLMM7 SCS treated HL-1 cells, but not in C2C12 cell. Moreover, Calcein AM staining showed decreased staining (indicating cell viability) in HL-1 cells treated with BGLMM7 SCS. No changes in Calcein AM staining were observed in C2C12 cells. Overall, according to the cell cycle profile, H3 (phospho S10), Cdk1 and CCNB1 expression, our data indicate strong potential of BGLMM7 SCS to block the proliferation of HL-1 cardiac muscle tumour cells. Moreover, this effect might be associated with Becn1 and autophagy induction. This preliminary research could be the basis for future research that would examine the potential of lactobacilli in treatment of soft tissue cancers, which are rare, but also of more common epithelial tumours both *in vitro* and *in vivo.* This offers the possibility of examination of eventual dedifferentiating effect of lactobacilli extracts, which could play significant role in regenerative medicine.

Results Unforeseen in the Original Project:

Interesting results arose also from this project and implicate a potential of *Lactobacillus sucicola* BGLMM7 SCS to suppress the proliferation and viability of tumour myocytes. In the same time, viability of non-tumour myocytes was not decreased after treatment with BGLM7 SCS, implying selective effect of SCS on tumour cells. This research opens the way for further analysis of effects of both bacterial SCS and WCE on proliferation of more common epithelial tumours both *in vitro* and *in vivo*. In addition, it indicated the potential of lactobacilli extracts to slow proliferation of non-tumour myoblasts (C2C12) without concomitant decrease of their viability. This offers the possibility of examination of eventual dedifferentiating effect of lactobacilli extracts, which could play significant role in regenerating medicine.

Publications:

Lukic, J., Dinic, M., Ruozi, G., Mirkovic, N., Djokic, J., Giacca, M., Strahinic, I., Begovic, J. Spent culture supernatant (SCS) from *Lactobacillus sucicola* BGLMM7 inhibits proliferation of tumour cardiac myocytes. 2016. J. Bacteriol. Parasitol. **7(6),** doi.org/10.4172/2155-9597.C1.026

SLOVAKIA

Title: Molecular profiling of cancer: biomarkers of progression, therapeutics and predisposition
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Abstract:
Prostate cancer is multifactorial disease and multiple single nucleotide polymorphisms (SNPs) could contribute to higher risk of prostate cancer development.
The study group consisted of 50 patients with prostate cancer (Gleason score ≥ 7) and 50 healthy men. Genome-wide association study was performed on Affymetrix Genome-Wide Human SNP Arrays 6.0. The results were analysed by two statistical approaches. The association analysis of individual SNPs was performed by PLINK 1.9 software package. The random forest analysis ranks SNPs according to their predictive power and their joint effects on the phenotype.
We identified 7 SNPs, four of them located in genes (rs12136562, rs41439745 - LOC101929023, rs9426908 - PRRX1, rs12328643 - REEP1, 9423252 - ACADSB, rs1465512, rs2826099), to be significantly associated with prostate cancer by PLINK analysis. The random forest analysis identified other 5 important SNPs, four of them located in genes (rs17002954, rs7554507 – LINC00302, rs1992675 – SHANK2, rs7746701 – FRMD1, rs7049614 – VGLL1).
Genome-wide association study represents the important tool for identification of multiple SNPs associated with susceptibility to prostate cancer. Using different statistical approaches for interpretation of GWAS results helps to select potential markers appropriate for further study.
Objectives:
Cancer prognosis is one of the most difficult areas of medicine. Stage diagnosis and therapy selection are but probabilistic choices the clinician makes because uncertainties cannot be eliminated. Great optimism that emerging technologies such as DNA microarrays would yield molecular biomarkers for prognostics has driven extensive research over the past decade. In cancer, especially advanced disease, genetic alterations often take the form of duplicated (or triplicated, etc.) and deleted DNA sequences. The result is that too much or too little of a given gene is present, leading to the dramatic cellular changes seen in tumour cells. Cataloguing these gene copy number alterations, and identifying the specific genes involved, might lead to an improved understanding of cancer and point us towards developing improved therapeutic agents.
Our hypothesis is that chromosome regions (genes) of "extra" DNA sequence copy number are, (1) undiscovered by conventional, lower-resolution techniques, and (2) contain possible cancer genes that might be used as "markers" or therapy targets in treatment. We aim to test and develop this hypothesis by using a new technique, based on DNA microarrays. DNA microarray technology is revolutionising many aspects of biology and medicine by allowing measurement of thousands of genes simultaneously. Microarray data analysis has rapidly progressed to be the point where useful extraction of data from microarray experiments is becoming routine, however the interpretation of the underlying biology is complicated by the staggering complexity in which the large numbers of studied genes could be structurally and functionally related. In this project we plan to extend this approach to map single nucleotide polymorphisms (SNPs) and copy number variations by using DNA microarray chips. The advantage is that thousands of genes can be assayed simultaneously, and any identified gene can be immediately located using data from the Human Genome Project. We plan to use 50 individuals (prostate cancer patients and healthy controls) blood samples to obtain a database of genetic changes. Using a genome-wide association study (GWAS) we will identify novel SNPs associated with increased prostate cancer risk, a phenotype with strong attributable risk for prostate cancer mortality and morbidity. Taken together we will have a list of potential oncogenes (i.e., genes increased in cancer) and tumour suppressors (i.e., genes lost in cancer progression) for further study.
Results Obtained:
For statistical analysis of results we used two different approaches. The first was traditional association analysis of individual SNPs using PLINK 1.9 software package. Another approach we used was selecting SNPs that may have effect on the disease by random forest method.
The PLINK 1.9 is a whole genome association analysis toolset (http://pngu.mgh.harvard.edu/purcell/plink/), which enables complex analysis of the results, from SNP filtering to association analysis, result annotation and creating gene reports. As the first step of the statistical analysis, we focused on association of individual SNPs with the risk of prostate cancer development. In addition to effect sizes estimates (per allele odds ratios (OR) and 95% confidence intervals (95% CI)), we estimated also two-sided p-values. We applied a significance level of $p < 0.05$. Adjustment was also made for multiple testing using Bonferroni correction.

After Bonferroni correction we found 7 SNPs to be significantly associated with prostate cancer. They are summarised in the table below.

	Chromosome	OR (95% CI)	Fisher´s exact p	Fisher´s exact Bonf. p	Gene
rs12136562	1	7.41 (3.47-15.85)	4.53E-8	0.036	-
rs41439745	1	0.06 (0.02-0.19)	1.96E-9	0.002	LOC101929023
rs9426908	1	10.5 (4.3-25.59)	1.22E-7	0.01	PRRX1
rs12328643	2	8.03 (3.59-17.95)	2.51E-8	0.02	REEP1
rs9423252	10	7.84 (3.51-17.5)	3.24E-8	0.025	ACADSB
rs1465512	12	0.04 (0.01-0.19)	1.02E-8	0.008	-
rs2826099	21	9.48 (4.07-22.08)	1.27E-8	0.01	-

Four SNPs significantly associated with prostate cancer were located in genes: LOC101929023, PRRX1, REEP1 and ACADSB.
Random forest analysis enables to rank SNPs according to their predictive power and their joint effects on phenotype. In contrast to the association analysis, no clear criteria exist to determine which SNPs should be selected for downstream analyses. For this purpose, we used recurrent relative variable importance measure (r2VIM) for selection of relevant SNPs. SNPs with score more than 2 were considered important and these should be the most predictive SNPs. The random forest analysis identified other 5 important SNPs, four of them located in genes (rs17002954, rs7554507 – LINC00302, rs1992675 – SHANK2, rs7746701 – FRMD1, rs7049614 – VGLL1). Association of these SNPs with prostate cancer was not yet described as well as their functional impact.

SLOVENIA

Title: Understanding the molecular basis of infection by oncogenic human papillomaviruses
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Abstract: Human Papillomaviruses (HPV) are a major cause of cancer-related death in women worldwide. The HPV L2 capsid protein plays an essential role during the early stages of viral infection, but the molecular mechanisms underlying its mode of action remain incompletely understood. The project is addressing the role of HPV and HPV L2 protein in utilisation of the host sorting machinery, particularly the adaptor protein Sorting Nexin 17 (SNX17). We showed that L2-SNX17 interaction occurs through NPxF/Y binding domain, with additional upstream and downstream sequences involved in stabilisation of the interaction. This interaction can be blocked by short L2 peptides consisting SNX17-binding domain. Characterisation of the spatio-temporal role of SNX17 in HPV infection revealed that HPV-16 L2 interaction with SNX17 occurs very early during infectious entry and that this appears to play a critical role in the subsequent completion of capsid disassembly and dissociation of L1 from the L2-DNA complex. We also showed that one consequence of the L2-SNX17 interaction is the recruitment of other components of the cellular sorting machinery, the small GTPase Rab4. While L2-SNX17 interaction is indispensable for the initial HPV infection, it does not have a major role in the later stages of the HPV life cycle and virion production. We also provided evidences that L2-SNX17 interaction perturb normal functions of SNX17.
Objectives:
In this project we aimed to determine how L2 and HPV utilise SNX17 for viral infection and to determine whether this represents a valid target for therapeutic or prophylactic intervention in HPV infections. The following objectives were proposed:
(i) Biochemical and functional characterisation of the L2-SNX17 interaction. Specifically, we addressed the sequence requirements for SNX17 recognition in L2 capsid proteins, effect of L2 inhibitory peptides on HPV infection, functional characterisation of the L2 interaction with SNX17 during the HPV infection and effect of L2 on the normal function of SNX17.
(ii) To analyse the role of SNX17 during viral maturation.
Results Obtained:
(i) Using a proteomic approach, we previously identified SNX17 as a strong interacting partner of HPV-16 L2. This interaction occurs through NPxF/Y binding domain, although we showed here that additional upstream and downstream sequences are necessary for the stabilisation of the interaction. Short L2 peptides consisting SNX17-binding domain can efficiently block L2-SNX17 interaction *in vitro*, thus representing a tool for inhibiting infection with HPV. However, we did not observe a consistent inhibition of HPV infection in HaCaT cells *in vivo* using a lipid-based cell delivery system. This is most likely related to inefficient delivery of L2 peptides into the cells. We foresee the major drawback of this approach for the *in vivo* applications in the currently unreliable and low efficient systems for the delivery of short peptides into the cytoplasm of living cells.
Characterisation of the spatio-temporal role of SNX17 in HPV revealed that the HPV-16 L2 interaction with SNX17 occurs very early during infectious entry, with a peak of interaction at 2 hours post-infection (hpi). The SNX17 recruitment was significantly higher with wild type PsVs compared to PsVs containing a mutant L2 that was defective in SNX17 recognition. We also looked at the ratio of HPV-16 PsVs colocalising with SNX17-positive vesicles during the course of HPV infection and we observed that wild type PsVs stayed in SNX17-positive vesicles up to 4 hpi, while the ratio of SNX17-mutant PsVs significantly dropped after 2 hpi. Immunoprecipitation experiments confirmed the interaction between HPV-16 L2 and SNX17 during virus entry, with a peak of interaction at 2 hpi, but with lower levels of association being maintained for at least 9 hpi.
Furthermore, we found evidence of L2-SNX17 association at the later stages of infectious entry, suggesting that SNX17-mediated sorting machinery is either involved at different stages of HPV trafficking or that L2-SNX17 interaction is a long lasting event in the HPV trafficking. The functional relevance of the potential transition of SNX17 to the perinuclear region remains to be determined.
The L2-DNA transition to the nucleus requires disassembly of the HPV capsid and dissociation of L1 from the L2-DNA complex. We were interested whether the ability of L2 to interact with SNX17 plays a role in these early steps of virus infection. Using conformational-dependent 33L1-7 antibody, we provided evidence that the L2-SNX17 interaction facilitates faster uncoating and dissociation of L1 from the L2-DNA complexes, although most likely in a non-SNX17 vesicular compartment.
Finally, we showed that one consequence of the L2-SNX17 interaction is the recruitment of other components of the cellular sorting machinery, the small GTPase Rab4. We observed that the

proportion of HPV-16 vesicles colocalising with Rab4 is high in the first 6 hpi, whilst this was not observed with the SNX17-binding deficient HPV-16 PsVs. Additional experiments are needed to confirm actual involvement of Rab4 in the HPV infection.
While the interaction between L2 and SNX17 appears essential for virus infection, we did not know whether L2 might directly affect the function of SNX17. This could be particularly important for the later stages of the viral life cycle when there are high levels of L2 protein expressed. To address this we investigated the effects of L2 overexpression on the internalisation and recycling of a known SNX17 cargo molecule, the LDL receptor (LDLR). Our results showed that there is no effect on the LDLR internalisation, however, recycling of LDLR to the cell surface is decreased in the presence of L2 protein. This part of the project is still ongoing and will be extended to other major recycling partners of SNX17.
(ii) It is well accepted that L2-SNX17 interaction plays an important role during viral entry, but it is possible that it plays a role also during the viral maturation process and in the HPV life cycle. We compared production of wild type PsVs and PsVs deficient in SNX17 binding, evaluating the yield, capsid protein content, DNA encapsidation, capsid stability and PsV infectivity. Our results showed that none of these parameters is affected by defective binding of L2 to SNX17 during the HPV production phase. These studies show that while L2-SNX17 interaction is indispensable for the initial HPV infection, it does not have a major relevance for the later stages of the HPV life cycle and virion production.
This project directly and indirectly supported training of University of Nova Gorica students at different levels. Research related to the project contributed to one PhD thesis and represent a major part of one diploma thesis and one master thesis, where the research is still ongoing.
The experiences gained in the course of the project contributed to a new grant application in collaboration with the Faculty of pharmacy, University of Ljubljana, and University medical centre Ljubljana-Division of Gynaecology and Obstetrics. We proposed to study the involvement of APOBEC proteins in the infection and oncogenesis of HPV viruses in cervical cancer.
In the future, we are planning to finish studies addressing effect of L2 on the normal function of SNX17 and to start investigating the role of APOBEC proteins in HPV infection and malignant cell transformation.

Results Unforeseen in the Original Project:

(i) Preliminary data on the role of APOBEC proteins in HPV infection;
(ii) Study addressing the effect of environmental factors on the infection with cutaneous HPV types.

Publications:

Broniarczyk, J., Bergant Marušič, M., Godzicka-Jozefiak, A., Banks L. Human papillomavirus infection requires the TSG101 component of the ESCRT machinery. 2014. Virology **460/461**, 83-90

Broniarczyk, J., Massimi, P., Bergant, M., Banks, L. Human papillomavirus infectious entry and trafficking is a rapid process. 2015. J. Virol. **89**, 8727-8732

Pim, D., Broniarczyk, J., Bergant, M., Playford, M.P., Banks, L. A novel PDZ domain interaction mediates the binding between human papillomavirus 16 L2 and sorting nexin 27 and modulates virion trafficking. 2015. J. Virol. **89**, 10145-10155

SOUTH AFRICA

Title: Unravelling the sorghum root microbiome: a route to enhancing crop performance

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Abstract: The rhizosphere, which is the narrow zone of soil that is influenced by root secretions, can contain up to 100 billion microbial cells per gram root and more than 30,000 prokaryote species; most of them unknown. In general, rhizosphere microbial communities are less diverse than those of the bulk soil because plant roots "select" for specific microorganisms to prosper in the rhizosphere.

There is increasing evidence that this "root microbiome" plays a pivotal role in determining plant health and productivity. Several studies have identified specific microorganisms responsible for this. However, those studies relied on small sample sizes and culture-dependent techniques. In contrast, exhaustive sampling and high-throughput sequencing hold enormous promise for discovering novel organisms that are drivers of processes relevant to plant health and productivity.

Sorghum is, after maize and wheat, the most important grain crop produced in South Africa. However, very little is known of the general microbiology, or of the relationships between its rhizosphere community composition, crop and soil types.

This project aimed to address this information deficit by undertaking an extensive sampling program of sorghum rhizosphere soils. Microbial community structure, composition and activity were linked to soil physicochemical properties.

Objectives:

(i) To pinpoint the environmental factors that better explain bacterial community composition and function;

(ii) To study the diversity of sorghum-rhizosphere bacterial communities;

(iii) To investigate the functional ability of sorghum-rhizosphere microbial communities;

(iv) To isolate bacterial strains with important plant growth-promoting activities.

Results Obtained:

We have documented bacterial communities in the rhizosphere of sorghum and sunflower grown in crop rotation in four different soils under field conditions. A comprehensive 16S rRNA-based amplicon sequencing survey revealed that the differences in alpha-diversity between rhizosphere and bulk soils were both crop and time dependent. Sorghum rhizosphere soils at flowering and senescence were more diverse than bulk soils, whereas sunflower rhizosphere soils at flowering were less diverse respect to bulk soils. Habitat type (rhizosphere vs bulk soil) and sampling time (plant growth stage) were important in explaining the variation in bacterial community composition (β-diversity) in soils grown with both crops. This was for both rhizosphere and bulk soil samples, implying that the temporal changes observed in the rhizosphere microbiome were both plant-driven and due to seasonal changes in the bulk soil biota. However, the influence of habitat type and sampling time was less important that the influence of soil type. In soils grown with sorghum, P and C concentrations had the strongest correlation with rhizosphere communities (ρ=0.21, P=0.001), whereas bulk soil communities were correlated with P, C and N (ρ=0.26, P=0.001). In soils with sunflower, the combination of K, Ca, Mg, P and C better explained rhizosphere communities (ρ=0.38, P=0.001), while bulk soil communities were better explained by K and P (ρ=0.51, P=0.001). Therefore, it seems that the rhizosphere communities correlated with a different set of soil parameters that the bacterial communities in bulk soil, which suggests that different factors shape these neighbouring communities.

The relative abundance of most bacterial phyla remained relatively stable through the sampling period, although the phyla *Proteobacteria* (in sorghum) and *Nitrospirae* (in sunflower) were enriched in the rhizosphere relative to the bulk soil. Several individual taxa were also relatively more abundant in the rhizosphere and/or found to be important in maintaining rhizosphere microbial networks. Interestingly, some of these taxa showed similar patterns at different sampling times, suggesting that the same organism may play the same or different functional/structural role at different plant growth stages and in different crops. By means of a culturing approach, we confirmed that some of these rhizosphere taxa had *in vitro* plant growth-promoting abilities.

The Analysis of functional genes and metabolic pathways revealed that the bulk soil and rhizosphere microbiomes of sorghum contained functionally different capabilities. Rhizosphere communities were potentially involved in several key functions such as the metabolism of carbon, nitrogen, phosphorus, sulphur and potassium compounds; as well as several adaptive functions towards environmental stressors and the supplementation of plant metabolic needs.

Overall, we have shown that rhizosphere and bulk soil communities are functionally different and identified prominent bacterial taxa that might be used to develop microbiome-based strategies for improving the yield and productivity of sorghum and sunflower under crop rotation.

Results Unforeseen in the Original Project:

As the sequencing cost has decreased dramatically in the last few years we could afford to increase our sample size and therefore to extend the study to a second crop (sunflower) grown after sorghum. This allowed us to study how rhizosphere microbial communities change in a crop rotation system.

Publications:

Oberholster, T., Vikram, S, Cowan, D., Valverde, A. Key microbial taxa in the rhizosphere of sorghum and sunflower grown in crop rotation. 2017. Sci. Total Environ. **624**, 530-539

VENEZUELA

Title: Molecular cloning and characterisation of metalloproteinases and disintegrins, with therapeutic potential, encoded by cDNAs from venom glands of Venezuelan *Bothrops colombiensis* snake

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Abstract: After receiving the ICGEB funds on May 2014, we travelled to the National Natural Toxins Research Center (Texas, USA), to carry out the training and the next experiments for the cDNA cloning of *B. colombiensis* gland venom. We constructed a cDNA library from the venom gland of *B. colombiensis*, and a set of 729 high quality expressed sequence tags (ESTs) was identified. A total number of 344 ESTs (47.19% of total ESTs) was related to toxins. The most abundant toxin transcripts were metalloproteinases (37.50%), phospholipases A_2s (PLA_2, 29.65%), and serine proteinases (11.92%). Minor toxin transcripts were linked to waprins (5.52%), c-type lectins (4.07%), ATPase (2.91%), cysteine-rich secretory proteins (CRISP, 2.33%), snake venom vascular endothelium growth factor (svVEGF, 2.33%), L-amino acid oxidases (2.03%), and other putative toxins (1.74%). While 160 ESTs (21.95% of total ESTs) coded for translation proteins, regulatory proteins, ribosomal proteins, elongation factors, release factors, metabolic proteins, and immune response proteins. Other proteins detected in the transcriptome (87 ESTs, 11.93% of total ESTs) were undescribed proteins with unknown function. The remaining 138 (18.93%) cDNAs had no match with known GenBank accessions. Then, we have started the expression of the gene 540, which expressed a disintegrin that it will explore in its function. Here, three new cDNAs encoding ECD disintegrin-like domains of metalloproteinase precursor sequences were obtained from a Venezuelan mapanare (*Bothrops colombiensis*) venom gland cDNA library, which had been cloned. Three different N- and C-terminal truncated ECD disintegrin-like domains of metalloproteinases named colombistatins 2, 3, and 4 were amplified by PCR, cloned into a pGEX-4T-1 vector, expressed in *Escherichia coli* BL21, and tested for inhibition of platelet aggregation and inhibition of adhesion of human skin melanoma (SK-Mel-28) cancer cell lines on collagen I. Purified recombinant colombistatins 2, 3, and 4 were able to inhibit ristocetin- and collagen-induced platelet aggregation. r-Colombistatins 2 showed the most potent inhibiting SK-Mel-28 cancer cells adhesion to collagen. These results suggest that colombistatins may have utility in the development of therapeutic tools in the treatment of melanoma cancers and also in thrombotic diseases.

Objectives:

(i) The construction a cDNA library from the venom gland of *B. colombiensis;*
(ii) The expression of disintegrins genes;
(iii) Testing of these disintegrins on platelet aggregation and antitumoral activity.

Results Obtained:

The cDNA library from a Venezuelan mapanare (*Bothrops colombiensis*) venom gland was constructed. Three new cDNAs encoding ECD disintegrin-like domains of metalloproteinase precursor sequences were obtained from this cDNA library. Three different N- and C-terminal truncated ECD disintegrin-like domains of metalloproteinases named colombistatins 2, 3, and 4 were amplified by PCR, cloned into a pGEX-4T-1 vector, expressed in *Escherichia coli* BL21, and tested for inhibition of platelet aggregation and inhibition of adhesion of human skin melanoma (SK-Mel-28) cancer cell lines on collagen I. Purified recombinant colombistatins 2, 3, and 4 were able to inhibit ristocetin- and collagen-induced platelet aggregation. r-Colombistatins 2 showed the most potent inhibiting SK-Mel-28 cancer cells adhesion to collagen.

Publications:

Suntravat, M., Uzcategui, N., Atphaisit, Ch., Helmke, T., Lucena, S., Sanchez, E.E., Rodriguez-Acosta, A. Gene expression profiling of the venom gland from the Venezuelan mapanare (*Bothrops colombiensis*) using expressed sequence tags (ESTs). 2016. BMC Mol. Biol. **17**, 7

Suntravat, M., Helmke, T., Atphaisit, Ch., Cuevas, E., Lucena, S., Uzcategui, N., Sanchez, E.E., Rodriguez-Acosta, A. Expression, purification and analysis of three recombinant ECD disintegrins (r-colombistatins) from P-III class snake venom metalloproteinases affecting platelet aggregation and SK-MEL-28 cell adhesion. 2016. Toxicon **122**, 43-48

VIET NAM

Title: Host induced gene silencing in the rice blast fungus *Magnaporthe oryzae*

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Abstract: The rice blast disease caused by *Magnaporthe oryzae*, is one of the most devastating threats to rice production worldwide. Many studies of rice blast resistance as well as of pathogenesis, infection mechanisms of *Magnaporthe oryzae* have been done previously by using genetic tools, but there are so much practical implications for the blast control using host-induced gene silencing (HIGS) in rice. This strategy can revolutionise the agricultural biotechnology, particularly in plant protection, has been considered as a potential approach recently for efficient control of infectious pathogens in various agronomic important crops. Recent studies of HIGS indicated sufficient trafficking of RNA molecules from host plants to fungal pathogens that were demonstrated successfully in fungal pathogens of wheat and barley such as *Blumeria graminis*, *Puccinia striiformis* f. sp. tritici. These results may put the solid background for HIGS applications in other crops against fungal pathogens, particularly in the rice blast fungus, *Magnaporthe oryzae*. Here, the main objective of this project is to create transgenic qualified rice varieties that can be resistant to rice blast fungus, *Magnaporthe oryzae*, by delivering HIGS constructs expressing hairpin double-stranded RNAs specific to fungal target genes resulting the reduction of fungal pathogenesis or infection structures formation of fungal pathogen.

Objectives:

(i) Establishing HIGS protocol for Indica rice varieties to suppress the expression of *Magnaporthe oryzae* CWDEs or some important calcium transcripts resulting the reduction of the development and infection process of the rice blast on the host plant. The success of this project will be helpful for application of HIGS on other important crops against the attacks of pest/pathogens;
(ii) Creating transgenic rice varieties against the blast disease;
(iii) Understanding HIGS approach for further studies to elucidate host-fungus interactions as well as for other application in controlling the attack of pest/pathogen on crops;
(iv) Training undergraduate-, graduate students to understand new concepts, techniques and applications of molecular biology in plant protection in particular and agriculture in general.

Results Obtained:

At least 30 *M. oryzae* isolates were collected from different rice fields in many provinces in Vietnam. Most of them were isolated from infected leaves and rice panicles The results of this experiment indicated that the conidial morphology of all 30 fungal isolates is similar to conidial morphology of the rice blast fungus, *Magnaporthe oryzae* with pear like shape and two septa inside the spores. These fungal isolates were identified using PCR reactions with Pot2 transposon and iDM primers (Harmon et al., 2003). The results showed that all fungal isolates showed similar bands of 687 bp and 548 bp corresponding the amplicons of Pots transposon and iDM respectively. The usage of specific *M. oryzae* primers such as Pot2 transposon and iDM plays an important role for in-field rapid detection of blast disease in rice as well as for reducing time and labour in identification of this fungal species compared to the morphology based methods. These fungal isolates were used as materials for further inoculation in the wild type and HIGS based transgenic rice lines.

Until now, many *M. oryzae* genes involving in fungal pathogenicity have been identified by using forward and reverse genetic techniques such as gene disruption, point mutation, RNAi and etc. In this study, the most important thing is to identify suitable pathogenicity genes of *M. oryzae* that can avoid causing off-target effects in transgenic rice lines. We already searched the entire rice genome for genes with homology to *M. oryzae* target genes such as calmodulin, *M. oryzae* Zn(II)2Cys6 transcription factor, F box protein, artificial DNA of endoxylanases and cellulases and etc. by using online searching databases. For this study, we selected two *M. oryzae* Zn(II)2Cys6 transcription factor genes (MGG_05343.6 and MGG_06086.6) of which one gene (MGG_06086.6) has not been characterised previously. *M. oryzae* Zn(II)2Cys6 transcription factor genes have been shown to be essential for development and pathogenicity of the rice blast fungus (Chung et al., 2013; Lu et al., 2014). MoCOD1 (MGG_05343.6) targeted mutants completely lost pathogenicity due to defects in appressorium formation and invasive growth within host cells (Chung et al., 2013). The second choice is the homolog of F box protein required for pathogenicity 1 *F. oxysporum f. sp. lycopercisi* (FPR1) (MGG_06351.6) containing F box domain that interacts with PKS1 to facilitate targeting proteins to the SCF-ubiquitination complex and is essential for *F. oxysporum f. sp. lycopercisi* pathogenicity due to unable colonisation to the host plants (Duyvesteijn et al., 2005). The third choice is the homolog to *Oryzae sativa* 12-oxophytodienoate

reductase (Tani et al, 2008) and the last one is *M. oryzae* MPG1 (MGG_10315.6) that is necessary for the pathogenicity of the rice blast fungus, *M. oryzae* (Talbot et al., 1993). We did not find any homolog or highly similar sequences of rice genome to those target genes and they are amplified from *M. oryzae* cDNA by PCR. We set up an alternative approach using artificial microRNA technology that can be genetically engineered and function to specifically silence single or multiple genes of interest in plants. In this technology, we focused on three targets of *M. oryzae* including MGG_05343.6, MGG_06086.6 and MGG_06351.6. By using web microRNA designer – WMD3, we identified potential miRNA sequences that can be cut-off at 10 first candidates. For constructing artificial miRNA construct, we will follow the protocol developed by Norman Warthmann, MPI Developmental biology, Germany. Those constructs will be made based on the vector pNW55. PCR products will be cloned into pGEMT vector for further experiments involving in transformation in rice. We successfully created HIGS constructs with these candidates and were in transformation in rice for selecting HIGS based transgenic rice lines based on molecular analyses.

Results Unforeseen in the Original Project:

Beside the original project, our group also got other results as the following:

(i) The virulence of 30 *M. oryzae* isolates was evaluated by inoculation in various rice varieties including 2 non-susceptible rice varieties (JHN from Thailand and IR50404 from Vietnam); 1 mediate blast resistance rice variety; 2 susceptible rice varieties (VĐ20 and KDML105). Although there are various levels of fungal pathogenicity, we found that at least 9 *M. oryzae* isolates were identified to have strong fungal virulence due to their breakdown of disease resistance in non-susceptible rice varieties in Thailand and Vietnam. The pathogenicity identification of those fungal isolates is very useful for further experiments, particularly in identification of markers involving in fungal virulence as well as for inoculation tests in transgenic lines of rice varieties used in this study.

(ii) The analysis of genetic diversity of blast fungi was carried out by RAPD, SRAP and ISSR methods. The aims of this experiment are not only to compare those methods but also to identify which method can provide more specific bands for the development of SCAR markers involving in fungal virulence. The results indicated that SRAP method showed the highest polymorphism and the phylogenetic tree analysis indicated that there are three separated groups of *M. oryzae* isolates, particularly clear distribution of the Northern group with other two groups. Interestingly, we also found some specific bands from three analyses that could be potential markers for fungal virulence.

(iii) We identify specific bands amplified by SRAP primers that could be developed SCAR markers for the identification of fungal virulence.

(iv) Screening the presence of avirulence genes from Vietnam rice blast fungus, *Magnaporthe oryzae.* The results of this experiments indicated that AVR-Pik and AVR-Pik CDS took the largest amount among isolated strains that were 96.67%. In contrast the M. oryzae isolates possess AVR-Pizt (which is the lowest) it was 36.67%.

(v) Sequence variation of avirulence AVR-PITA1 gene in Vietnamese rice blast isolates was analysed in this study. The phylogenetic analyses showed the diversity of Avr-pita1 sequences among 19 Viet Nam rice blast isolates. The data of this study can be used in further research related to rice blast resistant and breeding program in future.

(vi) Besides scientific outputs, the biggest impact of this project is to support us to train at least one Ph.D. candidate, 6 Master students of which two completed their Master program, 8 undergraduate and internship students. They were supported for their experiments and had opportunities for presenting their research results and/or learning experience in various national and international conferences. The most important thing of this project is to help us for improving research capacity for our students individually and Nong Lam University generally.

Publications:

Quoc, N.B., Chau, N.N The role of cell wall degrading enzymes in pathogenesis of *Magnaporthe oryzae.2016.* Curr. Protein. Pept. Sci. (in press)