CONVEGNO ANNUALE
DELLA SEZIONE LIGURE-LOMBARDO-PIEMONTESE
DELLA SOCIETA' ITALIANA DI BIOCHIMICA E BIOLOGIA MOLECOLARE

LLP 2011

20 maggio 2011
UNIVERSITA' DEL PIEMONTE ORIENTALE
"A.AVOGADRO"
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Aula Magna
Facoltà di Economia
Via Perrone
Novara

ABSTRACTS
ORAL
PRESENTATIONS

Biochemical characterization of *Plasmodium falciparum* CDPK4

Andrea Cavagnino[1], Menico Rizzi[1], Franca Rossi[1].

[1] DiSCAFF, University of Piemonte Orientale "A. Avogadro", Italy; cavagnino@pharm.unipmn.it

INTRODUCTION: Transmission of malaria requires an obligatory biological interplay between female *Anopheles* mosquitoes and a parasite of the genus *Plasmodium*, the etiological agent of the disease (1). *P. falciparum* Calcium Dependent Protein Kinase 4 (*Pf*CDPK4) is recognized as a molecular switch that translates the XA-induced calcium signal into a cellular response by regulating cell cycle progression (2). CDPKs family is characterized by the presence of a kinase domain, an autoinhibitory junction domain (J) and a C-terminal regulatory domain that is able to activate the enzyme following calcium binding (3).

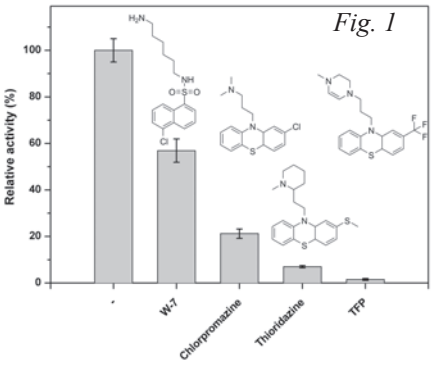

Fig. 1

RESULTS: The *Pf*CDPK4 open reading frame has been sub-cloned into the pET16b vector for the expression in *E. coli* of a His-tagged version of the kinase (*Pf*-HisCDPK4; predicted MW=63300.4 Da). *Pf*-HisCDPK4 has been purified to homogeneity through an affinity chromatography step followed by a size exclusion chromatography separation, which confirmed that the purified enzyme is present as monomer in solution. Non-radioactive, HPLC-based kinase assays revealed that monomeric recombinant *Pf*-HisCDPK4 is active on the substrate β-casein with a specific activity of 0.6 μmol min^{-1} mg^{-1}. *Pf*-HisCDPK4 can undergo auto-phosphorylation and its kinase activity strictly depended upon the presence of Ca^{2+} ions. The ATP utilization by *Pf*-HisCDPK4 displayed a classical hyperbolic kinetics with the Michaelis-Menten constants: V_{max}= 0.155•10^{-3} μmol/min, K_M=106.2 μM and k_{cat}= 48.9 min^{-1}.

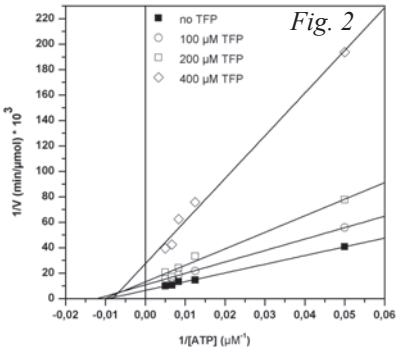

Fig. 2

The kinase activity has been measured in the presence of 1 mM concentration of the following molecules: W7, chlorpromazine, thioridazine and TFP (Fig. 1). The inhibition kinetics confirmed that TFP acts as an ATP non-competitive inhibitor of *Pf*-HisCDPK4 activity, displaying an apparent *Ki*=150 μM (Fig. 2).

Based upon available structural information, the analysis of the optimally superposed structures of the CaM/TFP complex (pdb:1CTR) and of the *T. gondii* CDPK1 in its activated Ca^{2+} state (pdb:3HX4) showed a severe collision between the inhibitor phenothiazine moiety and Leu339, a residue belonging to the C-terminal helix of the J domain of the kinase (Fig. 3), that we propose as the likely factor determining the inhibition exerted by TFP on *Pf*-HisCDPK4.

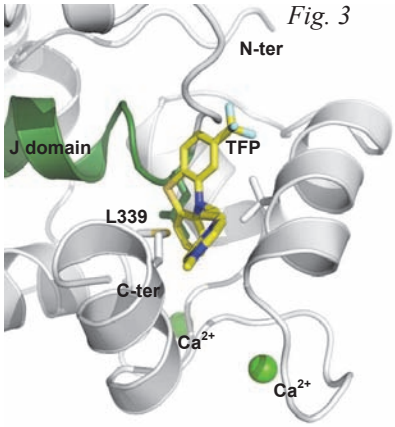

Fig. 3

In conclusion, we performed an enzymatic characterization of *Pf*CDPK4 using a non-radioactive method. Furthermore, by means of kinetic and structural analysis we identified *Pf*CDPK4 phenothiazine-based inhibitors. These last data highlight the CDPKs regulatory domain as a promising target for the development of innovative enzyme inhibitors.

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Malaria pigment hemozoin (HZ) and HZ-generated 4-hydroxynonenal inhibit malaria erythropoiesis

*Oleksii A. Skorokhod,[1] *Luisa Caione,[2] Tiziana Marrocco,[1] Giorgia Migliardi,[2] Valentina Barrera,[1] Paolo Arese,[1] Wanda Piacibello,[2] and Evelin Schwarzer[1]
*O.S. and L.C. contributed equally

[1]Department of Genetics, Biology and Biochemistry, and [2]Institute for Cancer Research and Treatment, IRCC Candiolo, Laboratory of Cell Therapy, University of Torino Medical School, Torino, Italy; olexii.skorokhod@unito.it

Keywords: malaria, lipoperoxidation, 4-hydroxynonenal, erythropoiesis

Severe malaria anemia (SMA) is a frequent complication and an important cause of mortality in children and pregnant women (1). SMA is characterized by inhibited/altered erythropoiesis and presence of hemozoin-(HZ)-laden bone-marrow macrophages. HZ mediates peroxidation of unsaturated fatty acids and production of bioactive aldehydes such as 4-hydroxynonenal (HNE, ref. (2, 3)).

HZ-laden human monocytes inhibited growth of co-cultivated human erythroid cells and produced HNE that diffused to adjacent cells generating HNE-protein adducts. Co-cultivation with HZ (Fig. 1) or treatment with low-micromolar HNE inhibited growth of erythroid cells interfering with cell cycle without apoptosis. Following HZ/HNE treatment, two critical proteins in cell cycle regulation, p53 and p21, were increased and the retinoblastoma protein, central regulator of G1-to-S-phase transition, was consequently hypophosphorylated, while GATA-1, master transcription factor in erythropoiesis was reduced. The resultant decreased expression of cyclin A and D2 retarded cell cycle progression in erythroid cells and the K562 cell line.

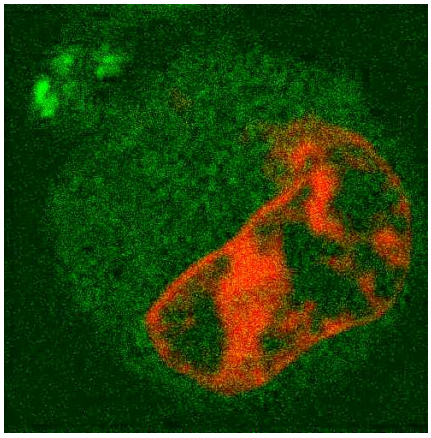

Fig. 1. Extracellular HZ increases the amount of HNE adducts on the cell surface of K562 erythroid cell co-cultivated with HZ (top left corner). In red is a cell nucleus stained with PI, in green are HNE adducts on cell surface.

As a second major effect, HZ and HNE inhibited protein expression of crucial receptors: transferrin receptor 1 (CD71), stem cell factor receptor (c-kit), IL-3 receptor (CD123) and erythropoietin receptor (EPOR). The reduced receptor expression and the impaired cell cycle activity decreased the production of cells expressing glycophorin-A and hemoglobin.

Present data confirm the inhibitory role of HZ, identify HNE as one HZ-generated inhibitory molecule and describe molecular targets of HNE in erythroid progenitors possibly involved in erythropoiesis inhibition in malaria anemia.

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The role of the focal adhesion kinase Pyk2 in platelet activation

Lina Cipolla, Ilaria Canobbio, Alessandra Consonni, Gianni Guidetti, Stefania Momi, Barbara Oliviero, Marco Falasca, Mitsuiko Okigaki, Cesare Balduini, Paolo Gresele, Mauro Torti

Department of Biochemistry, University of Pavia, Italy; Department of Internal Medicine, University of Perugia, Italy; Department of Infectious Diseases, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy; Queen Mary University of London, UK; Department of Cardiovascular Medicine, University of Kyoto, Japan

Keywords: focal adhesion kinase, murine model

INTRODUCTION: Pyk2 is a focal adhesion kinase, highly homologous to FAK, which does not possess SH2 or SH3 domains, but a centrally located catalytic domain flanked by a FERM domain and two proline-rich sequences. Pyk2 is unique in that it can be activated both by Src-mediated phosphorylation and by intracellular Ca2+ increase, and thus it can translate Ca2+ signals into a tyrosine kinase-based pathway (1). Pyk2 is highly expressed in platelets, and is rapidly activated by the majority of the physiological agonists, through an aggregation- and integrin αIIbβ3-independent mechanism (2). However, its role in platelet activation is still unknown. In this study we report a functional analysis of platelets from Pyk2-knockout mice (3).

RESULTS: Platelet count was normal in Pyk2 KO mice, and no significant differences in the expression of the major glycoproteins was observed by flow cytometry. In an in vivo model of photochemical-induced femoral artery thrombosis, the time for artery occlusion was significantly prolonged in the Pyk2 KO mice. In addition, Pyk2-deficient mice exhibited a marked protection against collagen plus epinephrine-induced pulmonary thromboembolism, with a significant reduction of the number of occluded vessels in the lung. Tail bleeding time was slightly increased in Pyk2 KO mice. Ex vivo analysis on Pyk2-deficinet platelets revealed that aggregation was normal upon stimulation of GPVI, but was almost completely suppressed in response to PAR4-activating peptide. Similarly, aggregation of Pyk2 KO platelets was strongly reduced in response to low but not high doses of thrombin or U46619. Thrombin-induced inside-out activation of integrin αIIbβ3, but not secretion was impaired in the absence of Pyk2. Finally, we found that Pyk2 was critically required for efficient TxA2 generation. These results reveal a novel important role for the focal adhesion kinase Pyk2 downstream of G-proteins coupled receptors to support platelet aggregation and thrombus formation.

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Activation by 2-arachidonoylglycerol of platelet p38MAPK/cPLA$_2$ pathway.

Maria Grazia Signorello, Enrica Giacobbe and Giuliana Leoncini

Department of Experimental Medicine, Biochemistry section, Genoa University, Genoa, Italy

The endogenous cannabinoid 2-arachidonoylglycerol (2-AG) is described as a platelet agonist able to induce aggregation and to increase intracellular calcium. In the present report we have confirmed these data and demonstrated that the inhibitor of p38MAPK SB203580 and the inhibitor of cPLA$_2$ metabolism ETYA affect both these parameters. Thus we aimed to define the role of p38MAPK/cytosolic phospholipase A$_2$ (cPLA$_2$) pathway in 2-AG-induced human platelet activation. p38MAPK activation was assayed by phosphorylation. cPLA$_2$ activation was assayed by phosphorylation and as arachidonic acid release and thromboxane B$_2$ formation. It was shown that 2-AG in a dose- and time-dependent manner activates p38MAPK peaking at 10 µM after 1 min of incubation. The 2-AG effect on p38MAPK was not impaired by apyrase, indomethacin or RGDS peptide but it was significantly reduced by SR141716, specific inhibitor of type-1 cannabinoid receptor and unaffected by the specific inhibitor of type-2 cannabinoid receptor SR144528. Moreover the incubation of platelets with 2-AG led to the phosphorylation of cPLA$_2$ and its activation. Platelet pretreatment with SB203580, inhibitor of p38MAPK abolished both cPLA$_2$ phosphorylation and activation. In addition SR141716 strongly impaired cPLA$_2$ phosphorylation, arachidonic acid release and thromboxane B$_2$ formation, whereas SR144528 did not change these parameters. Finally platelet stimulation with 2-AG led to an increase in free oxygen radical species. In conclusion data provide insight into the mechanisms involved in platelet activation by 2-AG, indicating that p38MAPK/cPLA$_2$ pathway could play a relevant role in this complicated process.

Identification of nucleotide-sugar biosynthetic pathways in the Nucleo-Cytoplasmic Large DNA Virus (NCLDV) mimivirus.

Francesco Piacente*, Parakkottil Chothi Madhu, Margherita Marin and Michela Tonetti

Department of Experimental Medicine, University of Genova, Viale Benedetto XV, 1 16132 Genova; tonetti@unige.it

Keywords: mimivirus, nucleotide-sugars

INTRODUCTION: Nucleo-Cytoplasmic Large DNA Viruses (NCLDV) comprise an heterogeneous group of viruses, characterized by very large genomes (0.3 to 1.2 Mbp) infecting several types of eukaryotic cells. Many evidences suggested that members of this group, in particular Chloroviruses and Mimivirus, encode at least in part, if not all, the glycosylation machinery required to glycosylate their structural proteins. This property differs from that of most viruses, which use the host ER/Golgi system for glycoprotein production and whose glycan structure and composition are completely dependent on their host cells. Several viral proteins involved in glycoconjugate formation have been already identified, including enzymes for nucleotide-sugar production (GDP-L-fucose and UDP-L-rhamnose), enzymes for hyaluronan synthesis and glycosyltransferases. Moreover, polysaccharide-degrading enzymes were also found.
Aim of this study is the characterization of the glycoconjugates from the giant mimivirus, which infects free-living Achantamoeba species. In particular we have analyzed the monosaccharide composition by GC-MS and we have then identified some of the enzymes encoded by mimivirus, which are involved in the production of the corresponding nucleotide-sugars.

RESULTS: GC-MS analyses of Mimivirus viral particles indicated that the major sugar components are rhamnose, glucose, N-acetylglucosamine and a monosaccharide not yet identified. Other sugars (fucose, arabinose, mannose and galactose) were found in lower amounts. The glycan structures are mainly associated with the long fibers which cover the viral particles. Infact, GC-MS analyses of the viral particles after proteolytic removal of the fibers revealed a significant decrease in sugar content. These glycan structures probably contribute to the Gram positive stain displayed by these virions. Identification of the constituent monosaccharides represented the first step for the further structural characterization of these glycans. Moreover, it has allowed us to identify other virally encoded enzymes, which are involved in modified sugar production and which are currently under study in our laboratory.
Studies on the enzymes involved in glycoconjugate production encoded by NCLDVs could provide important insights not only about their role in viral life cycles, but also, due to the long evolutionary history of these viruses, about the relationships with bacterial glycans and with the evolution of the eukaryotic glycosylation machinery.

New fluorinated sialic acid glycals with inibitory activity against *Vibrio cholerae* sialidase.

Paola Rota, Nadia Papini,* Pietro Allevi*, Roberto Mattina and Bruno Venerando*

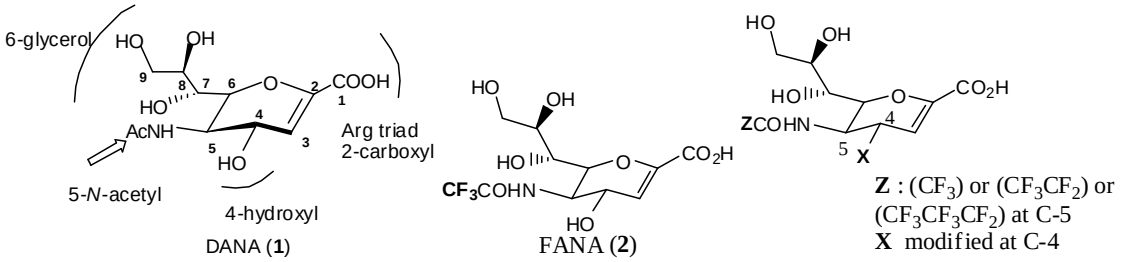
Department of Public Health, Microbiology, Virology, University of Milan, via Pascal 36, 20133 Milano Italy; paola.rota@guest.unimi.it

*Department of Chemistry, Biochemistry and Biotechnology for Medicine, University of Milan, 20133 Milano Italy.

Keywords: Sialic acid, Sialidase.

INTRODUCTION: The significant role played by sialidase in viral and bacterial infection has led to the widespread study of these glycohydrolases that cleave the terminal sialic acid at the non-reducing end of various glycoconjugates. In particular, influenza virus sialidase believed to play critical roles in the life cycle of the virus including the facilitation of virion progeny release, is an obvious target for the design of potential therapeutic agents. Based on some studies concerning viral, bacterial and human sialidases biochemistry, in previous papers we have reported the synthesis of some congeners of the glycal of sialic acid, 2-deoxy-2,3-didehydro-N-acetylneuraminic acid (DANA, **1**) a well known inhibitor of sialidases. In particular, we have found a simple protocol for the preparation of the perfluorurated analogue **2**, the 2,3-didehydro-2-deoxy-*N*-trifluoroacetylneuraminic acid (FANA, **2**),[1] having a ten times improved antimicrobial activity *in vitro* against *Vibrio cholerae*. Considering that the two sialidase inhibitors licensed for the treatment of influenza (Zanamivir and Oseltamivir) have the structure derived from DANA by substitution of the hydroxy group at C-4, we have developed some FANA derivatives having at position 4 a hindered aminic group.

RESULTS: We have set-up a short synthetic protocol, appropriately modulated, which allows to make any derivative of FANA bearing a different acyl group at the amidic group and a hindered substituent at C- 4.



The inhibitory activity of FANA congeners and its analogues substituted at carbon 4 were tested on *Vibrio cholerae* sialidase and compared with DANA and the commercially available Zanamivir by a fluorimetric assay using MU-Neu5Ac as substrate. Preliminary values of inhibitory activity (IC_{50}) show an increased activity (till 10 times higher) in respect to that of DANA. Rationalization of these and other results might allow to find additional insights on the steric requests of inhibitors to better fit with the catalytic site of the enzyme. Experiments are in progress to test the inhibitory activity of these compounds on mammalian sialidases and in particular on the human plasma membrane-associated sialidase, NEU3.

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Knockdown of *NEU4* during Zebrafish organogenesis

Giuseppe Paganini, Marta Manzoni, Daria Gotti, Giuseppe Borsani, Roberto Bresciani, Eugenio Monti

Department of Biomedical Science and Biotecnology, Faculty of Medicine, University of Brescia, Viale Europa 11, 25123 Brescia

Keywords: Enzymology, developmental biology

INTRODUCTION: Sialidases are enzymes that catalyze the removal of terminally linked sialic acid residues from glycoconjugates. In mammals four different sialidases have been identified and associated to several relevant processes such as lysosomal catabolism, regulation of cell adhesion, growth, differentiation, apoptosis and tumor transformation (1). In this study we focus our attention on NEU4, the intracellular membranes associated enzyme of the group (2). Human and murine proteins are expressed in two different isoforms (3). In particular murine *NEU4* seems to be involved, together with *NEU3*, in neural development and plasticity, including neurite formation (4). We used *Danio rerio* as animal model to investigate the biological role of NEU4 during embryonal development.

RESULTS: Through *in silico* analysis, based on the Zv9 Zebrafish genome assembly, we identified a ortholog gene in Zebrafish, named *neu4* (5). This gene is expressed from 8 hpf (hour post fertilization) to the adult organism. *In situ* hybridizations in 24 hpf-old-embryos show its expression within the marginal zone of the brain ventricle, while, starting from 48 hpf, the embryo labelling is restricted to the lens. RT-PCR demonstrates an almost ubiquitous expression in all tested organs, with highest levels of transcipt detectable in brain, eye, liver and kidney.
Knock-down experiments have been carried out using a splice-inhibiting Morpholino (MO N4i1e2) that leads to the skipping of the second exon, giving rise to a shorter transcript with an altered ORF.
A preliminary characterization of 24 and 48 hpf-old *neu4* morphants shows reduced cranial dimension and significant CNS disorganization, mainly involving the hindbrain and midbrain/hindbrain boundary and ventricular zone. This MO has been also injected in *Tg(kdr:EFGP)* embryos, and analysis of 30 hpf morphants revealed alteration in cranial vascularization and in the organization of intesegmental vessels.
We are currently performing further knock-down experiments using a different splicing-inhibiting MO in order to confirm the developmental defects.
Overall these results point out the relevance of neu4 in several relevant developmental processes, suggesting its pivotal role for the correct and complete development of the CNS.

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e-mail address: giuseppe.paganini@med.unibs.it

Proplatelet formation and extracellular matrices

Luca Monti [1], Cristian Gruppi [1], Alessandro Malara [1], Ruggero Tenni [1], Mario Raspanti [2], Cesare Balduini [1], Maria Enrica Tira [1], Alessandra Balduini [1]

[1]Dipartimento di Biochimica, Università di Pavia, Pavia Italy;
[2]Dipartimento di Morfologia Umana, Università dell'Insubria, Varese Italy;
cristian.gruppi@unipv.it

Keywords: megakaryocyte, collagen I.

Megakaryocytes (Mks) and their progeny, circulating platelets, are specialized mammalian cells that participate in haemostatic and inflammatory functions. Megakaryocyte differentiation and proplatelets formation (PPF) occur in the bone marrow and the mechanisms of these processes are still poorly understood (1). A number of evidence indicates that the nature of the microenvironment surrounding Mks may play an important role in the regulation of platelets production within the bone marrow (2).
Mks were differentiated from cord blood derived $CD34^+$ cells for 12 days. Mature Mks were plated on different collagen preparations or on different adhesive extracellular matrix proteins. Mk spreading and PPF were evaluated by phase contrast and fluorescence microscopy.
We found that adhesion of Mks to fibrillar type I collagen inhibited PPF, but not Mk spreading that was maintained over 16 hours incubation. This process was strictly dependent on fibrillar structure and biochemical properties of the adhesive substrate. In fact we showed that chemical modification of N-acetylation on collagen I, that leads to loss of negative charge on lysine residues and prevents "in vitro" fibrillogenesis, permitted PPF, while completely inhibited Mk spreading in 2 hour incubation (3).
Interestingly activation of Rho/ROCK pathway, upon engagement of $\alpha 2\beta 1$ integrin, occurred in Mks adherent on both collagen preparations. However, increased phosphorylation of myosin light chain 2 was observed in Mks adherent on native type I collagen with respect to Mks adherent on the modified collagen.
Therefore we hypothesized the existence of a reciprocal regulatory interaction between extracellular matrix structure and Mk function within the bone marrow microenvironment.

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Behavior of the plasma membrane associated glycohydrolases in Gaucher disease

Aureli Massimo*, Loberto Nicoletta*, Bassi Rosaria*, Filocamo Mirella°, Chigorno Vanna*, Prinetti Alessandro* and Sonnino Sandro*

* Departement of Medical Chemistry, Biochemistry and Biotecnology for the Medicine, University of Milano, via Fratelli cervi n°93, 20090 Segrate (MI) Italy; massimo.aureli@unimi.it
° S.S.D. Laboratorio Diagnosi Pre-Postnatale Malattie Metaboliche, IRCCS G. Gaslini, Largo G. Gaslini (GE), Italy

Keywords: Glycohydrolases, Sphingolipidoses, Plasma membrane

INTRODUCTION: Glycosphingolipids (GSL) are amphiphilic components of the outer layer of the plasma membranes acting not only a structural role but also as regulators of several proteins associated to the cell plasma membranes (PM) and, together with the PM proteins, they play an active role in the "cell social life"[1]. For several years in literature, beside to the complex intracellular sphingolipid metabolism, enzymes able to induce structural changes in the hydrophilic portions of the glycosphingolipid, working directly at the cell surface have been described [2]. Sphingolipidoses are lysosomal diseases, caused by the loss of one of the lysosomal sphingolipid hydrolase activity due to specific gene mutations [3]. Gaucher disease (GD) is due to the strong reduction of the β-glucocerebrosidase activity and three different clinical manifestation of the disease are known coded as Gaucher disease Type-1, Type-2 and Type-3. Until now no assays are available for the prognosis of the disease, and the treatments for most of the patients affected by this pathology results inadequate[4] .

RESULTS: Our experiments on PM associated glycohydrolases showed a surprisingly high conservation among all the different cell line tested of the activities assayed.
In particular, in patient affected by sphingolipidoses, we found for the enzyme involved in the pathology also a reduction of its PM associated activity. However comparing the total and PM activities in normal and pathological cells, we found that the residual activity in the PM was higher than that recovered in the total cells. In fibroblasts deriving from Gaucher disease type 1, 2 and 3 patients, we found that the CBE-sensitive β-glucosidase activity associated to the cell PM resulted down-regulated, whereas GBA2 resulted up-regulated with respect to the normal fibroblast. Moreover other PM associated activities resulted modulated, as β-hexosaminidases, β-galactosidases and arylsulphatases. Among the three sub-types of the Gaucher disease we found a different enzymatic profile of these PM associated activities.
We considered this information as the starting point to design new prognostic strategies for the different sub-type of Gaucher disease
In addition, new evidences on the relationship between the local pH conditions and the activity of PM associated glycohydrolases suggest that the use of drugs able to modulate PM enzymatic activities through the activation/inhibition of cell surface proton modulators could be a further innovative therapeutic approach to restore a more physiological enzymatic phenotype in GD cells.

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P2X7 antagonists improve “in vitro” myelination in organotypic dorsal root ganglia (DRG) cultures from a rat model of CMT1A neuropathy

Nobbio Lucilla*, Mannino Elena, Visigalli Davide*, Fulvia Fiorese*, Basile Giovanna, Sturla Laura, De Flora Antonio, Zocchi Elena, Schenone Angelo*, Bruzzone Santina

Dept of Experimental Medicine, Section of Biochemistry and CEBR, University of Genova, Genova, Italy; elena.mannino@hotmail.it
*Dept of Neurosciences, Ophthalmology, and Genetics and CEBR, University of Genova, Genova, Italy.

Keywords: P2X7, myelination

Charcot-Marie-Tooth 1A (CMT1A) is a hereditary neuropathy associated with overexpression of the peripheral myelin protein 22 (PMP22), causing demyelination. The molecular mechanisms leading to Schwann cell (SC) dysfunction are not understood and no treatment is available for CMT1A.
We reported an abnormally high intracellular Ca2+ concentration ([Ca2+]i) in SC from a rat model of CMT1A (CMT1A SC), caused by overexpression of the purinergic receptor P2X7. Correction of the elevated [Ca2+]i levels by the use of P2X7 antagonists or through down-regulation of P2X7 expression restored the normal phenotype in CMT1A SC.
We recently identified a new P2X7 antagonist, called P18, which is an isomer of Ap2A. P18 is also an agonist of P2Y11, the only purinergic receptor increasing the [cAMP]i, a positive regulator of SC differentiation.
Organotypic DRG cultures from both wt and CMT1A rats were treated for 3 weeks with 200 nM P18, or 1 μM A438079 (a commercially available P2X7 antagonist), or 1 mU/ml apyrase. All treatments significantly increase expression levels of the myelin protein MPZ (by 1.3-, 2.1- and 1.5-fold in CMT1A SC, by 1.4-, 1.1- and 2.3- in wt SC, respectively, as determined by western blots). Morphometric analysis of DRG, treated with P18 or A438079, and stained with Sudan black, confirms a significant increase of myelin segment density compared to untreated DRG. Neurofilament dephosphorylation levels, a measure of the possible detrimental effect on neurons, are not increased by the treatments.
The positive effect of P18 on myelination could also be mediated by P2Y11. Indeed, CMT1A SC show lower levels (by 40%) of the [cAMP]i compared with wt SC and addition of P18 (200 nM for 7 d) determines a P2Y11-dependent increase (1.6-fold) of the [cAMP]i in SC, as demonstrated by the use of a specific P2Y11 antagonist.
Therefore, P2X7 antagonist could represent a therapeutic strategy for CMT1A; the concomitant activation of P2Y11 could prove further advantageous.

LENTIVIRAL VECTOR-MEDIATED STRATEGIES TARGETED TO P38MAPK INHIBITION AND AKT ACTIVATION IN MOTOR NEURONS OF A MOUSE MODEL OF AMYOTROPHIC LATERAL SCLEROSIS.

Peviani Marco (a), Lidonnici Dario (a), Gensano Francesco (a), Tortarolo Massimo (a), Borsello Tiziana (a), Piva Roberto (b) and Bendotti Caterina (a)

a) *Dipartimento di Neuroscienze, Istituto di Ricerche farmacologiche "Mario Negri", Milano*
b) *Dipartimento di Patologia e Centro di Medicina Sperimentale (CeRMS), Università degli Studi di Torino*

Background. Amyotrophic Lateral Sclerosis (ALS) is a fatal progressive neurodegenerative disease characterized by selective death of motor neurons in the cortex, brainstem and spinal cord. This leads to weakness and muscular atrophy that evolves to paralysis and death. Mutations in the gene encoding for Superoxide Dismutase 1 (SOD1) account for approximately 2% of ALS cases. Studies on human ALS patients and animal models of ALS have evidenced that the pathology is the consequence of a complex interplay between several molecular pathways including excitotoxicity, mithocondrial dysfunction, accumulation of protein aggregates, proteasome dysfunction, altered axonal transport, neuroinflammation. Many of these mechanisms converge to the activation of common cell death signalling cascades in the motor neurons. In fact we and other groups demonstrated that in the motoneurons of either SOD1 mutant mouse model of ALS and patients with sporadic ALS there is a remarkable activation of the p38 mitogen activated protein kinase (p38MAPK) pathway. Increased activation of p38MAPK, in particular the alpha subunit, may induce hyperphosphorylation of neurofilaments leading to their accumulation in the perikarya, a hallmark of neurodegeneration. On the other hand, there are indications that motor neurons of ALS patients and transgenic mutant SOD1 mice are unable to trigger pro-survival signals such as the antiapoptotic PI3K/Akt pathway. We hypothesized that an unbalance between pro-degenerative (p38MAPK) and pro-survival (Akt) pathways in the motor neurons may account for the high vulnerability of these cells in ALS. To test this hypothesis we developed a lentiviral vector-based platform to selectively downregulate p38MAPK alpha through RNA interference (RNAi) and to activate Akt signaling pathway in vivo, in the motor neurons of mutant SOD1 mice.

Results. We demonstrated for the first time that a construct derived from Hb9 promoter can be used in lentivectors to restrict transgene expression to motor neurons in vivo; moreover we found that induction of Akt pathway, through expression of constitutively activated Akt3 in motor neurons, prevents neuronal loss in mutant SOD1 mice, although it does not influence their premature death. On the other hand, we used RNAi to downregulate p38MAPKalpha and revealed that expression of p38MAPK-targeted shRNA in the spinal cord of presymptomatic mice reduces motor neuronal loss in the early phases of the pathology, but then it is not able to sustain neuronal survival during disease progression, eventually resulting in worsening of symptoms and shortening of life span. We found that hyperactivation of microglial cells may be responsible for this more aggressive phenotype, suggesting that a fine tuning of microglial reactivity may be important to avoid pro-degenerative effects.

Conclusions. Overall these data support the hypothesis that modulation of pro-degenerative and pro-survival pathways may help counteracting motor neuronal degeneration, and strengthen the evidence that preservation of neuronal perikaria is not sufficient to ameliorate disease progression in ALS. The peripheral compartment (axons, muscles) should be regarded as an additional target for potential therapeutic approaches.

Structural and functional changes of bovine β-lactoglobulin after adsorption on hydrophobic surfaces: influence of the sorbent material and size

Matteo Miriani, Mauro Marengo, Marina Corrado, Stefania Iametti, and Francesco Bonomi

Dipartimento di Scienze Molecolari Agroalimentari, via Celoria 2, 20133, Milano, Italy.
matteo.miriani@unimi.it

Keywords: protein interface denaturation, allergenicity

INTRODUCTION: Adsorption of protein molecules on interfaces occurs in natural and man-made systems, and plays a central role in many events related to human health, to food science, and to environmental issues (1, 2). Structural rearrangements upon contact with the sorbent phase may affect the protein's biological activities (allergenicity, bioavailability, ability to bind micro- and macromolecular ligands). The surface characteristics of the sorbent material play a key role in the form and extent of these rearrangements. Knowledge of these phenomena may allow to predict or modulate the protein functional behavior after adsorption (3). In this work structural and functional changes undergone by bovine β-lactoglobulin (BLG) - a relevant food allergen - upon adsorption on hydrophobic surfaces in materials differing in nature and size (46 nm and 200 nm polystyrene nanoparticles, or oil-in-water microemulsions) were evaluated by means of intrinsic fluorescence spectroscopy, binding of fluorescent probes, accessibility of cysteine thiols, and limited proteolysis followed by MALDI-TOF and LC-MS identification of released peptides. Changes in immunoreactivity were evaluated by competitive ELISA using epitope-specific monoclonal antibodies.

RESULTS: Spectroscopic and reactivity studies indicate that the native structure of BLG undergoes extended stretching after adsorption on hydrophobic surfaces, exposing regions buried from the aqueous media in the native structure. Hydrolysis of BLG stuck on different surfaces results into different peptide patterns, but for a single peptide, likely a common region interacting with the sorbent phase. Immunoreactivity of BLG is also markedly altered upon absorption.

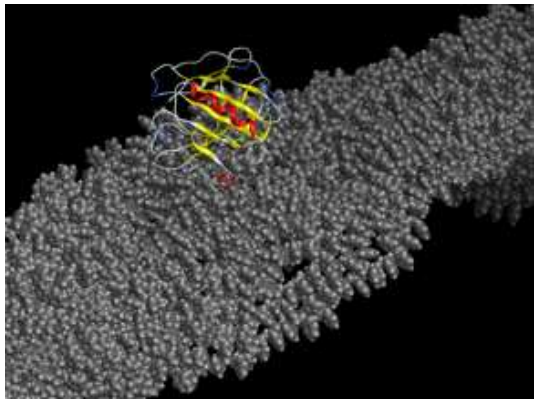

Fig. 1: Simulation of BLG denaturation on hydrophobic surfaces

The amplitude of the observed differences is depending on the nature and size of the sorbent material, that palys a central role in determining the type and extent of BLG structural rearrangements. *In silico* simulation surface denaturation is currently underway, and will be correlated with other evidences in order to pinpoint the regions most relevant to the various steps of the overall structural modification process.

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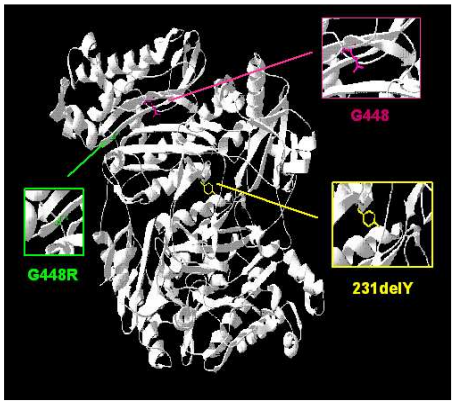
Insight on structural and kinetic properties of mutated human recombinant prolidase

Besio R.[1], Gioia R.[1], Monzani E. [2], Profumo A.[2], Nicolis S.[2], Casella L. [2] ,Rossi A.[1], Forlino A.[1]
[1]University of Pavia, Dept of Biochemistry; [2]University of Pavia, Dept of Chemistry
roberta.besio@unipv.it

Keywords: prolidase, prolidase deficiency, dipeptidase

INTRODUCTION: Prolidase (E.C.3.4.13.9) is an ubiquitous and cytosolic Mn II dependent dipeptidase. It is the only enzyme able to cleave the X-Pro, X-Hyp imidodipeptides, thus being very important in the final stages of protein catabolism and in particular of collagen turnover. It is a homodimer with two dimetal clusters. Mutations in the prolidase gene cause prolidase deficiency, a rare autosomal recessive connective tissue disorder characterized by severe and intractable skin lesions, mental retardation and respiratory infections.
We generated WT and mutated recombinant human prolidase in *E.coli*. We selected three mutations previously characterized in our patients: 231delTyr, Glu412Lys and Gly448Arg and causing, in homozygous condition, a strong reduction of enzyme activity. Tyr 231 is located at the N-terminal end in an alpha helix region, Glu412 is involved in the metal binding and Gly448, located in an extended strand, is very close to the metal binding site.

RESULTS: Mutated recombinant enzymes showed very low catalytic efficiency (24.01 $M^{-1}s^{-1}$ for 231delTyr, 1.37 $M^{-1}s^{-1}$ for Glu412Lys, 1.73 $M^{-1}s^{-1}$ for Gly448Arg) respect with WT (125.9 $M^{-1}s^{-1}$). V_{max} and K_M were evaluated for the WT enzyme (489 U/mg, 5.4 mM). Glu412Lys showed a low affinity for the substrate (38.1 mM) and a reduced V_{max} (28.9 U/mg); Gly448Arg normal affinity (5.9 mM) but a very low V_{max} (6.5 U/mg), whereas 231delTyr had a higher K_M (17.9 mM) in presence of a mild reduction of the V_{max} (261.5 U/mg).
The affinity constant for the cofactor Mn II was similar to WT (54.1 M^{-1}) in Gly448Arg, reduced in 231delTyr, higher in Glu412Lys (39 M^{-1}, 6.4 M^{-1}, 103 M^{-1} respectively).
ICP-MS analyses detected one Mn II ion in the WT active site following extensive dialysis. Interestingly two Mn II ions were detected in the Glu412Lys, suggesting that the nitrogen ligand coordinates the metal more strongly causing a rigidity in the active site probably responsible for the low catalityc efficiency detected.



CD analysis did not reveal any significant alterations in the secondary structure of the mutant proteins, but changes in the tertiary structure were demonstrated by limited proteolysis experiments. A delay in the dimerization process was detected in all mutated proteins; in particular Gly448Arg seemed to be in solution as a monomer at relative higher concentration. The Tm of the mutant enzymes was lower (52°C for Glu412Lys, 45°C for 231delTyr) with respect the WT (60°C) suggesting structural changes. A reduced prolidase amount in fibroblasts from PD patients, associated with normal mRNA levels, supported the presence of a structural alteration for 231delTyr and Gly448Arg.
The biochemical characterization of the three mutated prolidase allowed a better understanding of the molecular bases of the loss of activity in the PD patients carrying such malfunctioning enzymes.

This work was supported by Cariplo 2007 and MIUR 2008 to A.F.

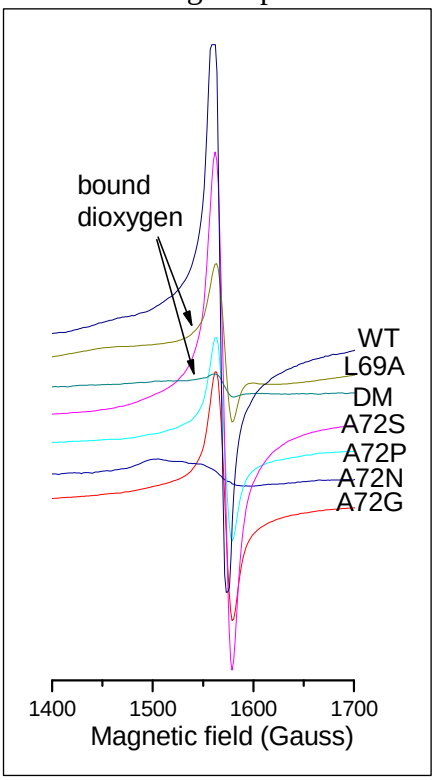
Mutations at position 69 and 72 in the catechol 1,2-dioxygenase sequence result in variants with distinct stability profiles and oxygen affinities

Raffaella Caglio[1], Francesca Valetti[1], Enrica Pessione[1], Carlo Giunta[1], Elena Ghibaudi[2]
[1]Dip. Biologia Animale e dell'Uomo, Università di Torino, Via Accademia Albertina 13, 10123 Torino
[2]Dip. Chimica Inorganica, Fisica e dei Materiali, Università di Torino, via Giuria 7, 10125 Torino

Keywords: catechol 1,2 dioxygenase, EPR spectroscopy, Fe(III) protein, mutagenesis.

INTRODUCTION: Several mutants of the B isozyme catechol 1,2-dioxygenase (IsoB C1,2O) [EC 1.13.11.1] from *Acinetobacter radioresistens* S13 have been expressed as a recombinant protein in *E.coli* BL21(DE3) and isolated. Mutations involved residues Leu69 and Ala72 and were chosen through structural alignments and active site homology modelling, based on the crystallographic structure of C1,2O from *A. calcoaceticus* ADP1 [1]. These two residues are extremely conserved in C1,2O from several distinct species and they are suspected to be involved in substrate recognition: in fact, their mutation has been shown to extend the range of potential C1,2O substrates, up to the inclusion of halogenated catechols [2]. Recently, the crystallographic structures of wt IsoB C1,2O and of some mutants have been resolved; based on the crystal structure, the catalytic pocket seems to be relatively conserved in all variants, although optical spectroscopy highlighted some differences in the iron environment [3]. In order to grasp additional information on the metal coordination sphere, C1,2O samples were also investigated by low-temperature EPR spectroscopy.

RESULTS: Fig.1 reports the EPR spectra collected at 4 K on samples of wt and mutant forms of C1,2O degassed with argon, as the original spectra denounced the presence of O_2 bound to the iron centre. O_2 was successfully removed in all samples, except L69A and the double mutant (L69A A72G), whose EPR traces agree with the presence of a strong magnetic interaction between O_2 and the metal centre. This may result from perturbations in the metal environment that reflect on the stability of the Fe-O_2 adduct and seem to be specific of these two mutants. In addition, EPR highlights a strong distortion of the iron environment in the A72N sample. Thermal stability studies, performed by optical spectroscopy by using the iron centre as a probe, show significant differences in the structural stability of the different mutants towards T. These measurements confirmed the behavioural analogy between L69A and the double mutant, as they both showed a similar and peculiar stability profile as compared with the other mutants. Finally, a distinct T-dependence of the enzyme activity was also found between C1,2O variants. The correlation of these experimental findings with the structural perturbations induced by mutations will be discussed.



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Intracellular protein O-GlcNacylation regulates cell microenvironment

Sara Deleonibus, Davide Vigetti, Manuela Viola, Evgenia Karousou, Giancarlo De Luca, and Alberto Passi

Dipartimento di Scienze Biomediche Sperimentali e Cliniche[1], Università degli Studi dell'Insubria, via J.H. Dunant 5, 21100 Varese, Italy, alberto.passi@uninsubria.it

Keywords: Glycobiology, Atherogenesis

INTRODUCTION: Changes in the microenvironment organization within vascular wall are critical events in the pathogenesis of vascular pathologies including atherosclerosis, restenosis and diabetic macroangiopathy. As the accumulation of HA into vessel wall supports neointima formation and cardiovascular diseases progression, we studied the intracellular regulation of HA synthesis and, in this work, we assayed whether its production could be controlled by nutrients availability.
Literature suggests that the excess of glucose could enter in the hexosamine biosynthetic pathway (HBP) that could increase the concentration of the HA precursor UDP-N-acetylglucosamine (UDP-GlcNac) leading to an increase of HA synthesis. In light of these issues the understanding of the mechanisms that regulate HA metabolism could represent new strategies to develop anti-atherosclerotic and vaso-protective drugs.

RESULTS: In this study, we tested the influence of different compounds to stimulate or reduce the flux through the HBP in the regulation of HA biosynthesis in human primary aortic SMCs (AoSMCs) that are known to be critical for neointima onset and progression. Besides to glycoconjugates, UDP-GlcNac can be the donor of GlcNac for O-GlcNacylation, a type of cytoplasmic protein O-glycosylation by which the monosaccharide GlcNac attaches to ser/thr residues via an O-linked glycosidic bond. We found that the inhibition of O-GlcNacylation strongly reduced HA production whereas treatments that induced protein O-GlcNacylation increased HA secretion. Gene expression studies done by quantitative RT-PCR revealed that HAS2 mRNA (i.e., the main HA synthesizing enzyme located in the cellular plasma membrane) was the most sensible to O-GlcNacylation and accumulated after its induction. Although factors governing constitutive HAS 2 transcription are yet to be established, we found evidences that the transcription regulator YY1 activates HAS2 expression after O-GlcNacylation. Interestingly, at protein level, experiments with wheat germ agglutinin, that specifically binds to GlcNac, and recombinant 6myc-HAS2 revealed that this enzyme was actually O-GlcNacylated. Further, HAS2 O-GlcNacylation increased HAS activity in purified microsomes leading to HA accumulation in culture medium.
Finally, as cell migration and adhesiveness are critical factors for neointima formation and progression, we quantified motility and monocytes binding in AoSMCs after different treatments and found that O-GlcNacylation increased cell invasion and inflammatory cell recruitment.
These data highlight the critical role of precursor availability and HBP to control cell microenvironment and behavior.

Study of the mechanism of action of LT175, a dual PPAR ligand that ameliorates lipid and glucose homeostasis and insulin sensitivity

M Giudici, F Gilardi[1], N Mitro, F Loiodice[2], G. Fracchiolla[2], A. Laghezza[2], G Pochetti[3], R. Montanari[3], A Lavecchia[4], U Guerrini[5], A Maggi[5], E De Fabiani, D Caruso, M Crestani

Lab. of Biochemistry & Molecular biology of lipids - Mass spectrometry "Giovanni Galli" Dept. of Pharmacological Sciences Università degli Studi di Milano; [1]University of Lausanne Center for Integrative Genomics Genopode Building; [2]University of Bari; [3]CNR, Montelibretti, Roma; [4]University of Napoli; [5] Dept. Of Pharmacological Sciences Università degli Studi di Milano

Peroxisome proliferator-activated receptors (PPARs) are ligand-dependent transcription factors that play a key role in the regulation of lipid and glucose metabolism. Recent works aim at developing dual PPAR α/γ agonists devoid of the side effects of the antidiabetic agents thiazolidinediones and the dual-agonists glitazars. We studied the molecular mechanism of action of a new compound, LT175, and the regulation of glucose and lipid metabolism in cell and animal models. In cell-based assays LT175 activates both PPARα and PPARγ. Because of the unique structural properties of the ligand-receptor complex, we studied the coregulator recruitment by FRET, which revealed that LT175 is a full PPARα and a partial PPARγ agonist. We tested the biological activity of LT175 in mouse adipocytes, showing lower lipid accumulation than the PPARγ full agonist rosiglitazone, which may be explained by the lower expression of genes for fatty acid uptake (CD36) and glycerol 3-phosphate formation (PEPCK and GYK), necessary for triglycerides accumulation. Using PPRE-LUC reporter mice we investigated the *in vivo* bioavailability of the compound, showing that LT175 switches on the PPAR-dependent transcription program in liver, white and brown adipose tissue. Administration of LT175 to Diet Induced Obese (DIO) mice, an animal model of insulin resistance, decreases plasma glucose, insulin, non-esterified fatty acids (NEFA), triglycerides and cholesterol while it increases adiponectin and FGF21 levels, ameliorating the metabolic profile and insulin sensitivity. LT175 decreases total body weight, lowering visceral fat as assessed by *in vivo* magnetic resonance imaging and increasing Brown Adipose Tissue (BAT) mass.
Data obtained by RT-qPCR in DIO and *db*/*db* mice show that LT175 enhances the expression of PPAR target genes in the liver and adipose tissue. LT175, opposite to rosiglitazone, does not increase the expression of renal sodium transporter ENaCγ, which is involved in fluid retention. These results indicate that LT175 has favourable effects on glucose and lipid metabolism with a reduction of some of the major side-effects induced by PPARγ agonists and could therefore represent a new PPAR ligand with a more favourable profile as compared to glitazones.
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A glycosphingolipid/caveolin-1 signaling complex inhibits the motility of human ovarian carcinoma cells

Cao Ting, Prinetti Alessandro, Illuzzi Giuditta, Prioni Simona, Aureli Massimo, Chigorno Vanna, Sonnino Sandro

Department of Medical Chemistry, Biochemistry and Biotechnology, University of Milan, Via Fratelli Cervi 93, 20090 Segrate, Milano, Italy; ting.cao@unimi.it

Keywords: Gangliosides GM3, Caveolin-1, c-Src, Motility, Ovarian cancer

INTRODUCTION: Recent works showed that GM3 ganglioside complexed with integrins controls tumor cell motility and invasiveness by regulating the activation state of c-Src. c-Src is activated in cells with low GM3 levels and high invasive potential, and artificially induced increase in GM3 levels caused inactivation of c-Src, influencing cell motility. On the other hand, it has been previously reported that caveolin-1 expression inhibits metastasis development and invasiveness in mammary tumor cells and promotes cell-cell adhesion in ovarian carcinoma cells by a mechanism involving inhibition of Src kinases.
In order to study the possible role of a multiprotein complex regulated by glycosphingolipids and organized by caveolin-1 in the transduction of signals through Src kinases in controlling tumor cell motility, we carried out a number of various investigations.

RESULTS: High GM3 synthase expression in A2780 human ovarian cancer cells resulted in 1) elevated ganglioside levels, 2) reduced in vitro cell motility and 3) enhanced expression of the membrane adaptor protein caveolin-1. The motility of low GM3 synthase-expressing A2780 cells was reduced in the presence of exogenous gangliosides and by Brefeldin A treatment (able to increase the cellular ganglioside levels). Treament of A2780 cells with gangliosides markedly increased caveolin-1 phosphorylation. In high GM3 synthase-expressing clones, both treatment with the glucosylceramide synthase inhibitor D-PDMP and transient silencing of caveolin-1 were able to strongly increase cell motility. c-Src plays a crucial role in controlling the motility of these cells: 1) the motility of low GM3 synthase-expressing cells was reduced in the presence of a Src inhibitor; 2) c-Src was less active in high GM3 synthase-expressing clones; 3) D-PDMP treatment of high GM3 synthase-expressing cells led to c-Src activation.
These data suggest a novel role for gangliosides in regulating tumor cell motility, by affecting the organization of a signaling complex organized by caveolin-1, responsible for Src inactivation downstream to integrin receptors, and imply that GM3 synthase is a key target for the regulation of cell motility in human ovarian carcinoma.

Atomic Force Microscopy study of lipid rafts in MDA-MB-231 human breast cancer cells after DHA incorporation

Paola A. Corsetto, Andrea Cremona , Francesco Orsini, Paolo Arosio, Ilaria E. Jovenitti, Stefania Zava, Gigliola Montorfano, Angela M. Rizzo

Dipartimento di Scienze Molecolari Applicate ai Biosistemi, Università di Milano, via Trentacoste 2, Milan, Italy. paola.corsetto@unimi.it

Keywords: Omega-3, Cancer, Lipid Rafts

INTRODUCTION: One of the major targets for breast cancer therapy is the epidermal growth factor receptor (EGFR). EGFR is a transmembrane protein with intrinsic protein tyrosine kinase activity that is activated by ligand binding, most important being EGF. EGFR over-expression contributes to increased cell proliferation and migration in breast cancer (1).
Recent findings in membrane biology suggest that the plasma membrane is composed by microdomains of saturated lipids that segregate together to form lipid "rafts". Lipid rafts have been operationally defined as cholesterol- and sphingolipid-enriched membrane microdomains resistant to solubilization by nonionic detergents at low temperatures. Lipid rafts are enriched in several signaling proteins, including EGFR (2).
(N-3) polyunsaturated fatty acids (PUFA), namely eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), decrease proliferation and induce apoptosis in EGFR over-expressing MDA-MB-231 human breast cancer cells (3).
Here we report a biophysical approach to investigate lipid rafts fatty acid and protein composition in MDA-MB-231 treated with DHA, applying spectroscopic and imaging tools, namely AFM and FTIR microspectroscopy. Moreover, the biophysical approach is coupled to a detailed biochemical analysis by means of biochemical assays (SDS-PAGE, Western Blotting and HPLC/GC).

RESULTS: Biochemical analyses show that DHA increases the unsaturated state of phospholipids in lipid rafts of breast cancer cells, therefore, alters their physical-chemical properties. Many acylated proteins directly interact with membrane lipid bilayers by their saturated acyl moieties. Then we suggest that altered lipid composition of microdomains might determine the displacement of proteins from lipid rafts in n-3 PUFA-treated cells with alteration of signal transduction, with particular regards to EGFR.
In addition, morpho-dimensional changes in lipid rafts are visualized and analyzed by AFM studying purified membrane samples both before and after the DHA incorporation. AFM technique allows to obtain three-dimensional images of the surface topography of lipid microdomains at nanometer resolution in a physiological-like environment thus providing structural/functional insights that cannot be obtained with more conventional approaches. High resolution AFM imaging shows on MDA-MB-231 lipid rafts, after DHA incorporation, features in agreement, for dimensions and shape, with membrane proteins. A more accurate investigation using specific antibodies could confirm, in the next future, the nature of the observed structures and allow their identification. These preliminary results suggest that AFM could be an useful tool to characterize changes in the membrane protein content induced by DHA treatment at single protein level.

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Nitric oxide and P-glycoprotein modulate the phagocytosis of colon cancer cells.

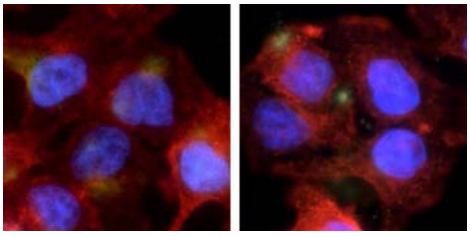
Joanna Kopecka, Ivana Campia, Sophie Doublier, Dimas Carolina Belisario, Dario Ghigo, Amalia Bosia, Chiara Riganti

Department of Genetics, Biology and Biochemistry and Research Center on Experimental Medicine (Ce.R.M.S.), University of Torino, via Santena 5/bis, 10126 Torino Italy; joanna.kopecka@unito.it

Keywords: doxorubicin (DOX), calreticulin (CRT), multi-drug resistance (MDR), nitric oxide (NO)

INTRODUCTION:
In recent years chemoimmunotherapy has been proposed as a powerful approach against advanced and disseminated cancers. A great interest has been arisen from the protocols based on doxorubicin (DOX), an anthracycline that elicits a direct cytotoxic death and an indirect pro-immunogenic death, by inducing the exposure on cell surface of specific immunosensitizing proteins. Among them, calreticulin (CRT), a chaperon and calcium sensor protein usually residing in endoplasmic reticulum, triggers the tumor recognition by local dendritic cells (DCs) [1]. We have previously demonstrated that chemoresistant tumors, that actively extrude DOX via the overexpressed membrane transporter P-glycoprotein (Pgp), do not undergo to citotoxic and to immunogenic death [2]. In this work we analyze the molecular mechanisms responsible for DOX-induced exposure of CRT and for the differences between chemosensitive and chemoresistant cells.

RESULTS: In chemosensitive colon cancer HT29 cells DOX increased the synthesis of nitric oxide (NO), that promoted the translocation of CRT on plasma membrane by activating sGC/cGMP/PKG/VASP pathway and remodelling actin cytoskeleton. CRT translocation did not occur in chemoresistant Pgp-overexpressing HT29-dx cells, where the NO synthesis was absent. By increasing NO with stimuli other than doxorubicin, the CRT exposure was obtained also in HT29-dx cells with the same mechanism. However, whereas in sensitive cells the CRT translocation was followed by the tumor phagocytosis, in drug-resistant cells the phagocytosis did not occur despite the CRT exposure. Indeed CRT physically interact with Pgp both in endoplasmic reticulum and in plasma membrane. Only by knocking-down Pgp protein, the DOX-operated phagocytosis was restored, suggesting that Pgp, when binds CRT, impairs the immunesensitization functions of the latter.



Calreticulin exposure after nitric oxide stimulation.
On the right side HT29 cells, on the left side HT29-dx cells, blue - nuclei, red - actin, green - calreticulin

In conclusion, our work shows that NO mediates the pro-immunogenic death of tumour cells inducted by DOX and that high amounts of Pgp causes not only chemoresistance, but also immunoresistance. The combination of conventional anticancer drugs like doxorubicin, with NO-releasing agents and Pgp-downregulating tools, can successfully reverse both chemo-and immunoresistance, appearing as a promising combination in chemoimmunotherapy protocols.

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In neuroblastoma cell lines saquinavir potentiates the down-regulatory effect of imatinib on the expression of the F-box protein Skp2.

E. Lupino, F. Timeus*, N. Crescenzio*, C, Ramondetti, A. Doria*, B Buccinnà, MT. Rinaudo, A. Tovo*, L. Cordero di Montezemolo*. M. Piccinini.
Departments of Medicine and Experimental Oncology, Section of Biochemistry and Pediatrics* University of Turin, Turin, Italy

INTRODUCTION

Metastatic neuroblastoma is an aggressive pediatric malignancy of the sympathetic nervous system. With current treatment protocols about 80% of high-risk patients will go into remission. However, despite aggressive multimodal therapy, the majority of these patients relapse and have long term survival rates of less than 40% (1). Amplification of the MYCN oncogene which characterizes around 25% of neuroblastomas is a clinical prognostic marker for poor survival. However a considerable number of MYCN-single copy neuroblastomas exhibit an aggressive phenotype similar to that of MYCN-amplified tumors even in the absence of high MYCN expression levels.

The F-box protein Skp2 is the limiting factor of the SCF E3 ligase complex which, by controlling the degradation of the cki $p27^{kip1}$, is critically involved in the regulation of cell cycle progression. Elevated expression of the F-box protein Skp2 characterizes high-risk neuroblastoma and Skp2 protein expression has been proven to be a highly significant marker of dire prognosis, independent of MYCN status and disease stage. Noteworthy, Skp2 protein expression was demonstrated to be inversely correlated with the expression of $p27^{kip1}$ in neuroblastoma tumors (2).

A number of observations indicate that c-kit, PDGFR and their ligands play a substantial role in the proliferation and survival of neuroblastoma cells (3, 4). Imatinib mesylate, an inhibitor of Abl tyrosine kinase, has been shown to inhibit the tyrosine kinase activities of c-Kit and PDGFR and neuroblastoma tumorigenesis in vitro. However in vivo it showed little or no activity as a single agent in children with relapsed or refractory neuroblastoma (5). HIV protease inhibitors (Pi) are widely utilized antiretroviral drugs which also show anti-cancer activity. PI anti-tumour effects have been documented on various tumour cell lines such as human prostate carcinoma, glioblastoma, leukaemia, non-small cell lung cancer and melanoma (6).

RESULTS

In the present study we compared the effects of imatinib alone or in association with the Pi saquinavir on the proliferation of two neuroblastoma cell lines: IMR-5 and SJ-N-KP. Both imatinib and saquinavir when used as single agents inhibited in a dose dependent manner neuroblastoma cell proliferation. Their effects added when the two drugs were used in combination. At the molecular level, the two drugs in combination down-regulated the expression of Skp2 independent of MYCN expression level.

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ELENA RICCITELLI - Abstract LLP 2011

Extracellular sphingosine-1-phosphate acts as a survival factor for human glioblastoma stem cells

E. Riccitelli, P. Giussani, L. Brioschi, G. Condomitti, C. Tringali, P. Viani, L. Riboni
Department of Medical Chemistry, Biochemistry and Biotechnology, University of Milan, LITA Segrate, Via Fratelli Cervi 93, 20090 Segrate, Milan, Italy

Keywords: Sphingosine-1-phosphate, glioblastoma stem cells

INTRODUCTION : Sphingosine-1-phosphate (S1P) is a sphingoid molecule formed by the phosphorylation of sphingosine in a reaction catalyzed by sphingosine kinases. S1P represents a key regulator of crucial processes in different cells, and several lines of evidence support its implication as an onco-promoter signal favouring growth, invasivity and survival of different cancer cells (1). S1P appears to be involved as an important mediator also in human glioblastomas, the most frequent and aggressive intracranial tumours (2). The use of the alkylating agent temozolomide in glioblastoma therapy has improved patient survival, but drug resistance mechanisms limit its therapeutic benefits. Accumulating literature indicates that glioblastoma stem-like cells (GSCs), a subpopulation of cells with the exclusive ability to self-renew and maintain the tumor, might contribute to tumor aggressiveness, and resistance to therapy (3). The aim of the present study was to investigate the possible role of S1P in the survival properties of GSCs. To this purpose we have established a GSC line derived from U87 human glioblastoma cells.

RESULTS: GSCs efficiently formed neurospheres in mitogen-defined medium and expressed the neural-stem cell markers CD133 and nestin. GSCs were found more resistant to temozolomide toxicity than parental U87 cells. Notwithstanding, the DNA repair protein MGMT, a major contributor to temozolomide resistance, was undetectable in both cell types. Interestingly, the exposure of GSCs to a sphingosine kinase inhibitor increased cell sensitivity to temozolomide, thus reducing drug resistance. In addition we found that S1P was present in the culture medium from both GSCs and U87 cells, and its level was significantly higher in the medium from GSCs than parental cells, thus suggesting that GSCs can efficiently produce/secrete S1P as extracellular mediator. Altogether our data implicate GSCs as an important source of exogenous S1P, which may contribute to their survival and invasive properties.

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Production of evolved variants of D-amino acid oxidase for a cancer enzyme therapy: a computational and biochemical study

Elena Rosini, Gianluca Molla, Sandro Ghisla and Loredano Pollegioni

Centro di Ricerca Interuniversitario in Biotecnologie Proteiche "The Protein Factory", Politecnico di Milano and Università degli Studi dell'Insubria; elena.rosini@uninsubria.it

Keywords: Protein engineering, oxygen reactivity, cancer therapy

INTRODUCTION: D-amino acid oxidase (DAAO) is a flavoenzyme that catalyses the oxidation of D-amino acids into the corresponding α-keto acids, ammonia and hydrogen peroxide (1). We previously proposed yeast DAAO in a enzyme-activating prodrug therapy of solid tumors, in which the *in situ* production of H_2O_2 by the flavoenzyme can be regulated by administration of D-amino acids. Unlikely, the *in vivo* application of DAAO was limited by the high K_m for this substrate and the low local oxygen concentration. In order to optimize DAAO for this application, at first we used a directed evolution approach (2) and, most recently, we combined a computational (Implicit Ligand Sampling) and a site-saturation mutagenesis approach (3, 4) to investigate the O_2 diffusion and reactivity in this flavoprotein.

RESULTS: The directed evolution approach allowed to isolate one DAAO mutant (m-DAAO, containing 5 point substitutions) with an increased activity at low O_2 and D-alanine concentrations and a 10-fold lower K_m for O_2. The observed higher activity resulted from a combination of modifications of specific kinetic steps, each being of small magnitude (2).
Moreover, several interconnected paths leading dioxygen from the solvent to two O_2 high-affinity sites (sites A and B, Fig. 1) close to the active site were identified (3). Site-saturation mutagenesis at three positions that flank the putative O_2 high-affinity sites was performed. These studies identified the T201L DAAO which shows a threefold increase in the rate constant for reaction of O_2, together with a fivefold decrease in K_m for dioxygen (3, 4).
Interestingly, a remarkable cytotoxic activity of DAAO plus D-alanine on mouse tumor cells was demonstrated, especially when the m-DAAO and T201L DAAO variants were employed.

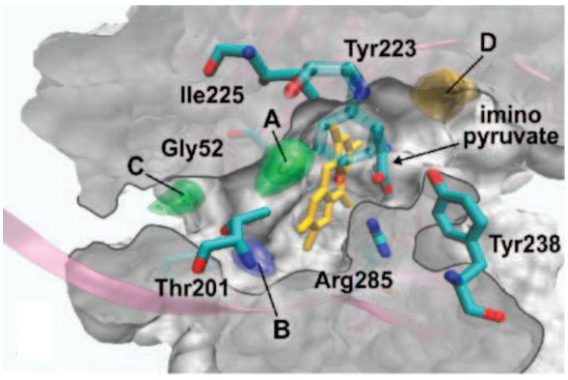

Fig. 1: Overview of O_2 access pathways to the active site of DAAO (3).

These results highlight the potential *in vivo* application of these evolved enzymes for tumor therapy. Moreover, the computational results provided a detailed mechanistic picture for the existence of functional oxygen channels in DAAO, a result of great interest for designing new, optimized flavoenzymes for biotechnological applications.

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Rab5 participates in chromosome congression by ensuring proper localization of CENP-F at kinetochores.

Gaetana Serio[1], Valentina Margaria[1], Sanne Jensen[2], Jiri Bartek[2], Federico Bussolino[1], and Letizia Lanzetti[1]

[1]Dipartimento di Scienze Oncologiche, Università degli Studi di Torino, Istituto per la Ricerca e la Cura del Cancro, Str. Provinciale 142 10060 Candiolo, Torino, Italy.
[2]Institute of Cancer Biology and Centre for Genotoxic Stress Research, Danish Cancer Society, Strandboulevarden 49, DK-2100 Copenhagen, Denmark.

Keywords: Rab5, mitosis

INTRODUCTION: Rab5 is a GTPase involved in the early steps of endocytosis and in cell motility. In its active GTP-bound form, it recruits downstream effectors that, in turn, are responsible for distinct aspects of early endosome function from signal transduction to selection and transport of cargoes (1).
Endocytosis is a complex cellular program through which cells regulate several aspects of their homeostasis including mitosis (2). Of note, Rab5C has been found in a mass-spectrometry-based spindle inventory (3), suggesting that also Rab5 might have a function at the spindle. However, the potential involvement of Rab5 in mitosis has not been addressed so far.

RESULTS: In this study, we unveil a novel function of Rab5 in chromosome congression. Our results show that RNAi-mediated silencing of Rab5 caused defects in chromosome congression and an extensive prometaphase delay. Analysis of Rab5-silenced cells revealed a decrease in the stability of kinetochore microtubules and a severe reduction in the localization of the centromere-associated protein CENP-F at kinetochores. CENP-F is required for chromosome congression, stable microtubule capture at kinetochores and for the localization of other outer kinetochore components such as the mitotic kinesin CENP-E. Simultaneous depletion of both Rab5 and CENP-F recapitulated the remarkable delay of anaphase onset, defective chromosome alignment and mislocalization of CENP-E that were comparable to those caused by silencing of either Rab5 or CENP-F indicating epistatic roles for both proteins in a pathway that orchestrates chromosome congression and cell division.

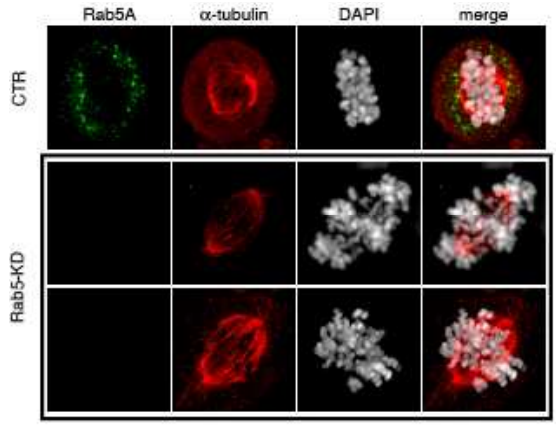

Fig. 1. Chromosome uncongression in Rab5-KD cells.

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Analysis of the action mechanism of small compounds displaying Ras inhibitory properties

Elena Sacco[I], Elisa Mazzoleni[I], Annalisa D'Urzo[I], Stefano Lamperti[I], Luca DeGioia[I], Maria Samalikova[I], Rita Grandori[I], Vadim Gaponenko[II], Francesco Peri[I] and Marco Vanoni[I]

[I]Department of Biotechnology and Biosciences, University of Milano-Bicocca, Italy
[II]Department of Biochemistry and Molecular Genetics, University of Illinois at Chicago, USA

INTRODUCTION: Ras GTPases cycle between inactive GDP-bound state and active GTP-bound state to modulate a diverse array of processes involved in cell proliferation, differentiation, migration and survival (1). Ras−GTP levels *in vivo* are controlled by the competing activities of Guanine nucleotide Exchange Factors (GEFs) that, by catalyzing the nucleotide-bound dissociation on Ras, promote GDP/GTP exchange inducing Ras activation and GTPase Activating Proteins (GAPs) that, by increasing the intrinsic GTPase activity of Ras, promote the hydrolysis of bound-GTP leading to Ras inactivation. Mutations of *RAS* genes are critical events in the pathogenesis and progression of different human tumours and Ras proteins represent a major clinical target for the development of specific inhibitors to use as anticancer agents (2).

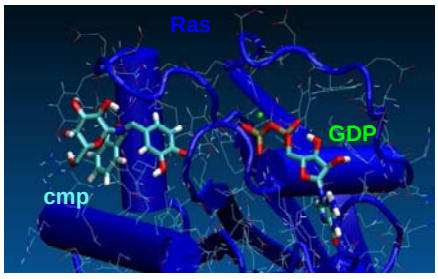

Fig.1. Docking model of Ras-GDP-Compound complex

RESULTS: Recently water-soluble Ras inhibitors were synthesized (3,4,5) and *in vivo* experiments show that the addition of compounds in the culture medium of normal and *kras*-transformed fibroblasts induces a dose-dependent decrease in proliferative potential. The compounds inhibit in a dose-dependent manner GEF-catalyzed dissociation of the guanine nucleotide from Ras and the entrance of the new nucleotide (5).

The action mechanism of compounds is being analyzed in detail on both wild type and mutant Ras using both computational (docking and molecular modelling) and biochemical and biophysical data including calorimetric assays (ITC), SPR and mass spectrometry experiments.

By using a computer model describing the possible interaction of the compounds with the multiple step Ras/GEF cycle, that involves binary and ternary complexes between Ras, guanine nucleotide, and GEF [based on a model of the Ras/GEF cycle published by Lenzen et al., 1998 (6)] we are evaluating alternative models for the action of the compounds. The choice of unknown parameters for the simulations is constrained by above described experiments. This integrated computational and experimental system-level approach should prove valuable in fine characterization of drug mechanism and in the development of novel drugs with improved s electivity and /or efficacy.

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2D TR-NOESY NMR Experiments Interrogate and Rank Ligand–Receptor Interactions in Living Human Cancer Cells

Silvia Mari, Chiara Invernizzi*, Andrea Spitaleri, Luca Alberici*, Michela Ghitti, Claudio Bordignon*, Catia Traversari*, Gian-Paolo Rizzardi* and Giovanna Musco

Biomolecular NMR Laboratory, Dulbecco Telethon Institute c/o S. Raffaele Scientific Institute, Via Olgettina 58, 20132 Milano, mari.silvia@hsr.it
*MolMed SpA, via Olgettina 58, 20132 Milano

Keywords: NMR, integrin αvβ3, aminopeptidase N, angiogenesis

INTRODUCTION: Integrins, the major class of heterodimeric transmembrane glycoprotein receptors, and the membrane-spanning surface protein aminopeptidase N (CD13), play a pivotal role in tumour growth and metastatic spread, as they are two of the major membrane bound receptors highly expressed on the surface of tumour cells during angiogenesis, therefore gaining increasing importance as drug targets in antiangiogenic cancer therapy.
Recent biochemical studies have shown that the deamidation of the NGR sequence gives rise to isoDGR, a new αvβ3-binding motif[1]. Accordingly, a cyclo-peptide containing the isoDGR motif is a competitive antagonist of αvβ3-ligand RGD, inhibiting endothelial cell adhesion, proliferation, and tumour growth. No competition is observed with cyclo-peptides containing DGR or NGR sequences, thus implying a precise stereospecific recognition at the basis of isoDGR interaction with αvβ3[2].

RESULTS: Here we show that it is possible to apply 2D-TR-NOE techniques directly on human cancer cells to prove selective binding of anti-angiogenic ligands to structurally characterised and uncharacterised receptors, such as αvβ3 and APN (CD13), respectively[3]. We investigated the binding of a small library of cyclopeptides (CRGDC, CisoDGRC, CDGRC, CNGRC, cyclo(-RGDfV-) onto 2 human cancer cell lines differently expressing αvβ3 and CD13, including a melanoma (MSR3:αvβ3+CD13- cells) and a non-small lung carcinoma (MR300:αvβ3+CD13+) cell line, which display different phenotypes for CD13 and integrins. Only very small amount of receptors are needed to prove binding (in the picomolar range), as it is sufficient that the receptor is detectable by FACS analysis. The method allows using different cell lines, with different receptors, which can be also silenced with siRNA techniques to prove recognition specificity. Moreover, we show that 2D-TR-NOESY can be applied in living tumour cells to screen varying ligands to the same receptor, performing competition experiments thus defining an affinity ranking of different ligands in a physiological context.

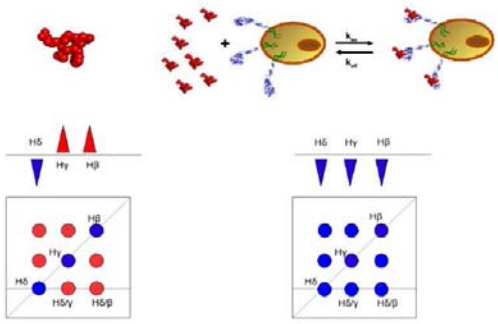

Fig. 1. Illustration of 2D-TR-NOE technique

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Semaphorin 3A blocks tumor growth and invasiveness and prevents the resistance to conventional anti-angiogenic therapies by overcoming cancer hypoxia

Federica Maione[1,2], Stefania Capano[1,2], Donatella Regano[1,2], Lorena Zentilin[3], Mauro Giacca[3], Federico Bussolino[2], Guido Serini[2,4] and Enrico Giraudo[1,2]

Laboratory of [1]Transgenic Mouse Models and [4]Cell Adhesion Dynamics, Institute for Cancer Research and Treatment (IRCC), Candiolo, Italy
[2]Department of Oncological Sciences, University of Torino, School of Medicine, Candiolo, Italy.
[3] Molecular Medicine Laboratory, International Centre for Genetic Engineering and Biotechnology (ICGEB), Trieste, Italy
Keywords: Tumor angiogenesis, Antiangiogenic therapy

INTRODUCTION: Class 3 Semaphorins (Sema3), are implicated in the regulation of both physiological and tumor angiogenesis. We have recently shown that endothelial Sema3A is an endogenous inhibitor that is lost during tumor progression and its reintroduction into a mouse model of pancreatic islet β-cell carcinogenesis (RipTag2), resulted in reduced vascular density, blood vessel normalization, restoration of tumor normoxia, and inhibition of tumor growth (1).

RESULTS: We demonstrate that the treatment of tumor-bearing RipTag2 with exogenous Sema3A induced a dramatic reduction of tumor invasiveness, the reappearance of E-cadherin and a down-modulation of vimentin, two known targets of cancer hypoxia that are also regulated during epithelial-mesenchymal transition (EMT). Then, we sought to investigate if the preventive administration of exogenous Sema3A was able to overcome the evasive resistance observed in RipTag2 upon treatment with Sunitinib, an anti-angiogenic agent that inhibits several tyrosine kinase receptors (2). Notably, we observed a dramatic reduction of tumor volume, cancer invasiveness, liver and peripancreatic lymph node metastases in RipTag2 treated for 2 weeks with Sema3A followed by 2 weeks of Sunitinib treatment, compared to Sunitinib-treated controls. Moreover, while Sunitinib-treated tumors were highly hypoxic and displayed few pericyte-covered vessels, the combinatorial regimen of Sema3A and Sunitinib normalized the vasculature and restored tumor normoxia. Finally, Real-Time RT-PCR and confocal microscopy analysis revealed a strong increase of E-cadherin expression and a complete inhibition of vimentin in tumors treated with Sema3A and Sunitinib as compared to Sunitinib alone.
Therefore, re-expression of Sema3A in tumors may safely harness the therapeutic potential of anti-angiogenic drugs, by normalizing the vasculature, inhibiting tumor hypoxia, and modulating the expression of EMT markers and other hypoxic-induced genes activated by anti-angiogenic treatments.

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Increased $A\beta_{42}$ peptide production in brain capillary endothelial cells after oxygen and glucose deprivation

Alessandra Bulbarelli, Elena Lonati, Anna Brambilla, Antonina Orlando, Emanuela Cazzaniga, Fabrizio Piazza[1], Carlo Ferrarese[1,2], Massimo Masserini, Giulio Sancini

Department of Experimental Medicine, University of Milano-Bicocca, via Cadore 48, 20900 Monza (MI), Italy. a.brambilla18@campus.unimib.it

[1]Department of Neuroscience and Biomedical Technologies, University of Milano-Bicocca, 20900 Monza, MI, Italy
[2]Department of Neurology, San Gerardo Hospital, 20900 Monza, MI, Italy

Keywords: Alzheimer's Disease; amyloid-β 1-42; Amyloid beta-Protein Precursor; Blood-Brain Barrier; Brain Ischemia-Anoxia; HIF-1alpha protein

INTRODUCTION: Although the diverse triggers of Alzheimer's disease (AD) are still under debate, in recent years the hypothesis of cerebrovascular deficiencies contribution has emerged [1]. Indeed cerebrovascular insufficiencies, such as reduced blood supply to the brain or disrupted microvascular integrity, might occupy a significant role in neurodegenerative events during AD onset [2,3].
A possible contribution of vascular deficiency in AD neurodegenerative mechanisms might be the aggregates of toxic amyloid-β (1-42) ($A\beta_{42}$) constituting senile plaques, one of AD hallmarks, often detected as amorphous material or fine fibrils in brain capillary of AD patients [4,5]. The $A\beta_{42}$ source in brain capillary is still not clear, however it has been proposed that it might origin either from the circulating blood, the brain parenchyma or the vessel wall itself [5].
Recent studies in neuronal cells demonstrated that hypoxia increased expression and activity of beta-secretase 1 (BACE1) [6], the main protease catalyzing amyloidogenic cleavage of amyloid-β protein precursor (APP), suggesting the hypothesis that hypoxic conditions might elicit $A\beta_{42}$ production also in brain capillary endothelial cells.
For all above mentioned, we subjected rat immortalized brain capillary endothelial cell RBE4, to oxygen glucose deprivation (OGD), as an *in vitro* model of the ischemic cerebrovascular condition investigating the increase in $A\beta_{42}$ production.
RESULTS: In the present investigation we show, that in rat brain capillary endothelial cells (RBE4), *in vitro* oxygen glucose deprivation (OGD) treatment elicits an increase of $A\beta_{42}$ peptide production through a mechanism that involves the hypoxia inducible factor-1 (HIF-1)-mediated BACE1 up-regulation. Furthermore, we observed a time dependent increase of amyloid protein precursor (APP) gene and protein expression.
In conclusion, our experimental evidences suggest that brain capillary endothelial cells may directly contribute to the amyloidogenic metabolism.

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Diacylglycerol kinase alpha produced phosphatidic acid controls myosin light chain kinase localization and activity in HGF stimulated epithelial cells.

Baldanzi[1], Birolo[2], Malacarne[1], Pighini[1], Pagnozzi[2], Chianale[1], Filigheddu[1], Sinigaglia[1], Pucci[2], Graziani[1]

1 Dep. Medicina Clinica e Sperimentale, Novara, Italy and BRMA, Novara, Italy
2 Dep. Organic Chemistry and Biochemistry, Naples, Italy

Diacylglycerol kinases (DGKs) regulate lipid signaling by phosphorylating diacylglycerol to phosphatidic acid (PA). Membrane recruitment and activation of DGK-alpha (DGKα) is required for cell proliferation, migration and matrix invasion induced by growth factor, chemokines and oncogenes in epithelial and endothelial cells.

Through a proteomic approach we identified 76 proteins associated to tyrosine phosphorylated DGKα in Kaposi sarcoma cells. Among these proteins we selected non-muscle myosin light chain kinase (MLCK), for further characterization. MLCK is a regulator of acto-myosin driven contraction, which phosphorylates myosin light chain (MLC). DGKα co-immunoprecipitates with both long (non-muscle) and short (smooth muscle) MLCK isoforms, consistently with the interaction observed with the proteomic strategy.

Inhere we show that in epithelial cells:

i) HGF-induced activation of DGKα produces PA at the nascent ruffle;

ii) DGKα-mediated PA generation is necessary and sufficient to recruit MLCK at ruffling site were MLCK promotes ruffle extension;

iii) expression of a constitutively active and membrane-bound DGKα mutant induces ruffles formation by recruiting MLCK at plasma membrane and promoting MLCK-mediated MLC phosphorylation;

iv) expression of constitutively active MLCK is sufficient to lead to ruffle assembly independently from DGKα activity.

Altogether those data indicate that PA produced by HGF-induced activation of DGKα, is a novel regulator of the localization and the activity of MLCK required for ruffle formation in epithelial cells.

ONLINE ABSTRACTS

Short-and long-term gonadectomy affects neuroactive steroid levels in the central and peripheral nervous system of male and female rats

Abbiati F.[#], Pesaresi M.[°], Maschi O.[#], Giatti S.[°], Giavarini F.[#], Garcia-Segura L.M.[§], Cermenati G.[#,*], De Fabiani E.[#], Crestani M.[#], Mitro N.[#,*], Melcangi R.C.[°], Caruso D.[#].

[#]Laboratory of Biochemistry and Molecular Biology of Lipids and Mass Spectrometry "Giovanni Galli", Dept. of Pharmacological Sciences, Università degli Studi di Milano, Via Balzaretti 9, 20133, Milano, Italy; fax: +39-0250318391 e-mail: federico.abbiati@unimi.it.
[*]Giovanni Armenise-Harvard Foundation Laboratory, Dept. of Pharmacological Sciences, Università degli Studi di Milano, Milano, Italy.
[°]Department of Endocrinology, Pathophysiology and Applied Biology (DEFIB), "Center of Excellence on Neurodegenerative Diseases" (CEND), Università degli Studi di Milano, Milano, Italy.
[§]Instituto Cajal, C.S.I.C., Madrid, Spain.

Keywords: Neuroactive steroids, nervous system, gonadectomy, LC-MS

INTRODUCTION: Significant levels of neuroactive steroids are still detected in the nervous system of rodents after the removal of peripheral steroidogenic glands. However, the influence of the plasma levels of gonadal steroids on those of neuroactive steroids in the nervous system has not so far been clarified in detail.
To this aim we have analyzed, by liquid chromatography tandem mass spectrometry, the levels of neuroactive steroids in the sciatic nerve, in three CNS regions (i.e., cerebellum, cerebral cortex and spinal cord) and in plasma of male and female animals.

RESULTS: The levels present in short- and long-term gonadectomised animals were compared with those present in gonadally intact animals. We observed that: (I) changes in neuroactive steroid levels in the nervous system after gonadectomy do not necessarily reflect the changes in plasma levels; (II) long-term gonadectomy induces changes in the levels of neuroactive steroids in the peripheral nervous system (PNS) and the CNS that, in some cases, are different to those induced by short-term gonadectomy; (III) the effect of gonadectomy on neuroactive steroid levels is different between the PNS and the CNS and within different CNS regions; and (IV) the effects of gonadectomy on neuroactive steroid levels in the nervous system show sex differences. Altogether, these observations indicate that the nervous system adapts its local levels of neuroactive steroids in response to changes in gonadal hormones with sex and regional specificity and depending on the duration of the peripheral modifications.

SEARCHING FOR NEW OMEGA-OXIDANT BACTERIA: A STUDY ON *ACINETOBACTER RADIORESISTENS* S13

Zapponi M., Fattori P., Mazzoli R., Pessione A., Giunta C. and Pessione E.
Università degli Studi di Torino, Lab. Proteomica e Biochimica dei Microrganismi, DBAU

Introduction

Biodegradable plastics can find applications in several commercial products, so-called "environmental friendly". In the present research we try to obtain biodegradable polymers from vegetable oils, low-cost industrial by-products that constitute a renewable source of fatty acids (FA). The limiting step of FA polycondensation is the lack of a second carboxyl or hydroxyl function in a terminal (ω) or subterminal (ω-1) position. We employed an aromatic-degrading *Acinetobacter radioresistens* S13 strain whose hydroxylating ability, via both mono- and di- oxygenases, has been previously proved (Pessione *et al*., Eur. J. Bioch., 2003). The growth of *A. radioresistens* S13 was firstly tested on several aliphatic hydrocarbons (C9, C10, C12, C16, C18) as the sole carbon source. The strain proved to be able to metabolize them, with growth different kinetics, suggesting the presence of an ω-oxidative enzyme system. Then we tested growth on fatty acids (from C9 to C18) obtaining satisfactory results for C9 and C18: considering these results and the availability of industrial wastes, we chose pelargonic acid as the most interesting substrate. Experiments are underway to inhibit catalytically, or by gene deletion, β-oxidative enzymes in order to obtain ω-hydroxy and ω-carboxy fatty acids

Results

We set up a gas chromatography method to verify the consumption of carboxylic acids and the parallel formation of metabolites of interest, with the goal to determine the best conditions for future comparative proteomic experiments. After that we evaluated the consumption of pelargonic acid when it was present as the sole carbon source and when it was supplemented with acrylic acid (β-oxidation inhibitor) and acetate as second carbon source. Results show complete metabolization of pelargonic acid despite the β-oxidation inhibitor.

We also performed a comparative proteomic experiment in order to evaluate different protein expression with pelargonic acid as sole carbon source in comparison with acetic acid, that do not enter in β-oxidation. Preliminary results from protein identification must be integrated with another proteomic experiment in which cells will be grown with the two organic acid and acrylic acid.

Characterization of a yeast strain with improved copper resistance

G. M. Adamo[a], S. Brocca[a], S. Passolunghi[a], M. J. Tamás[b], and M. Lotti[a]

[a] Università degli Studi di Milano-Bicocca – Dept. Biotechnology and Bioscience, Milano, Italy
[b] Göteborgs Universitet – Dept. Cell and Molecular Biology, Göteborg, Sweden

Keywords: Evolutionary adaptation, copper resistance, oxidative stress

INTRODUCTION: Copper is an essential micronutrient participating as a cofactor in redox reactions and is therefore important for the function of many proteins (1). The same redox properties that make copper an essential trace element are also responsible for its toxicity. Indeed, copper participates in chemical reactions generating reactive oxygen species (ROS) causing cellular damage (2). As copper is both essential for life and highly toxic for all organisms, homeostatic mechanisms, such as pathways for Cu uptake, distribution and detoxification, have been evolved to prevent both its excess and deficiency (3,4). Copper metabolism is tightly regulated and studies on simple model systems, like yeast, contribute to the comprehension of mechanisms involved in metal homeostasis in higher eukaryotes (5).

RESULTS: In this work, an evolutionary engineering approach was applied to obtain a copper hyper resistant *Saccharomyces cerevisiae* strain. Wild type cells exposed to increasing copper concentrations resulted able to tolerate up to 2.5 g/L $CuSO_4$. The features of the hyper resistant strain resulted stably maintained and consisting of copper accumulation, lower rate of mortality and reduced ROS production. To gain insight into the molecular basis of copper tolerance, we assessed the involvement of Cup1, a low molecular weight metallothionein that binds copper and mediates copper resistance in yeast cells. Compared to the wild type strain, the evolved one showed a 7-fold increase of *CUP1* copy number and a corresponding increase of its expression.
This work points out the potential of the evolutionary engineering approach to obtain strains with improved stress robustness and confirms the usefulness of yeast as a eukaryotic model to investigate on copper metabolism.

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An approach to understand the glycosphingolipid-protein interactions

Giuseppina Brasile,[1] Federica Compostella,[1] Laura Mauri,[2] Riccardo Casellato[2], Fiamma Ronchetti[1] and Sandro Sonnino[2]

Dipartimento di Chimica, Biochimica e Biotecnologie per la Medicina, Università di Milano

[1]Via Saldini 50, 20133-Milano, Italy; [2]Via Fratelli Cervi 93, 20190- Segrate (Milano), Italy

E-mail: giuseppina.brasile@unimi.it

Keywords: glycosphingolipid, photoactivable probes, signal trasduction

INTRODUCTION: Lipid-rafts are membrane microdomains particularly enriched in glycosphingolipids (GSLs) and proteins involved in trasduction processes across the membrane.
GSL-protein interactions can be investigated by cell photolabelling experiments using radioactive photoactivable GSLs, which yield, when illuminated, a very reactive intermediate that covalently binds to the molecules in the environment, *i.e.* proteins (1,2). The preparation of a GSL probe with two photoactivable groups, one at position 2 and the other at the end of the acyl chain of ceramide, could be a tool for the simultaneous identifications of the proteins involved in the biological recognition and belonging to both the extracellular and the cytoplasmatic leaflets of the membrane.

RESULTS: The preparation of an α,ω-diaminoacid as a bifunctional fatty acid useful for the conjugation of GLSs to photoactivable groups, *i.e.* nitrophenylazide, through the amino functionalities has been performed. This lipid chain has been conjugated to a radioactive *lyso*-ganglioside GM_1, tritium labeled at position 3 of sphingosine, obtained by an enzymatic reaction with a SCDase. The purpose is to verify the capability of this new kind of probes to enter the cells and participate to metabolic processes. So, the photolabelled ^{3}H-GM_1 will be administer to cells and its metabolic fate will be studied.

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In search of new mitochondrial regulators: genome-scale screening and functional profiling

Brioschi E.[*,#], Cermenati G.[*,#], Saez E.[§], Caruso D.[#], Crestani M.[#], De Fabiani E.[#], Mitro N.[*,#]

[*]Giovanni Armenise-Harvard Foundation Laboratory, Department of Pharmacological Sciences, Università degli Studi di Milano, Via Balzaretti 9, 20133 Milano, Italy; elisabetta.brioschi@unimi.it
[#]Laboratory of Biochemistry and Molecular Biology of Lipids and Mass Spectrometry "Giovanni Galli", Department of Pharmacological Sciences, Università degli Studi di Milano, Milano, Italy
[§]Dept. of Chemical Physiology, The Scripps Research Institute, La Jolla, California, USA

Keywords: Mitochondrial regulators, Genome-scale screening

INTRODUCTION: Mitochondria catalyze numerous biosynthetic and oxidative reactions fundamental to cell function[1] and generate most of the energy in animal cells[2]. As these organelles are ubiquitous, their density and function have a significant impact on whole-body metabolism. Finding new mitochondrial regulators could expand the knowledge on cell and tissue biology and biochemistry. Up to the present, only few transcription factors or coactivators (e.g. PGC-1α, NRF-1, mTFA) have been associated with mitochondrial biogenesis. Thus the aim of the present study was to find new potential factors able to control mitochondrial density and activity.

RESULTS: Through a genome-scale mTFA-based high-throughput screening in HEK 293 cells, starting from two cDNA libraries accounting for 70% of known genes, it was possible to identify clones able to induce and to reduce mTFA promoter activity, considering PGC-1α as positive control and p160 myb binding protein as negative control. The positive clones were then validated for their ability to modulate mitochondrial density and function using staining assays (Mitotracker™ Green and Mitotracker™ CM-H2-Rox). As a result, 200 positive hits were confirmed to induce these parameters at least as much as PGC-1α. Finally, these genes were classified for their biological and molecular function using a bioinformatic analysis in order to identify the biological pathways in which they are involved. The understanding of the role of new mitochondrial regulators could help identify new targets for the development of future interventions for the treatment of metabolic diseases associated to mitochondrial dysfunction.

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Glucose-induced increase of Abscisic Acid (ABA) levels in human plasma and ABA-stimulated glucose uptake by adipocytes

Santina Bruzzone, Pietro Ameri[*], Lucia Briatore[*], Giovanna Basile, Annalisa Salis, Elena Mannino, Sonia Scarfì, Gabriella Andraghetti[*], Giovanni Murialdo[*], Renzo Cordera[*], Antonio De Flora, Elena Zocchi
Dept. of Experimental Medicine, Section of Biochemistry, and Center of Excellence for Biomedical Research, University of Genova, Italy; santina.bruzzone@unige.it
[*]Dept. of Endocrinological and Medical Sciences, University of Genova, Viale Benedetto XV, 16132 Genova, Italy.

Keywords: Abscisic acid; glucose metabolism

The plant hormone abscisic acid (ABA) has been demonstrated to be released from glucose-challenged rodent and human pancreatic beta cells. Moreover, nanomolar concentrations of ABA stimulate both glucose-dependent and -independent insulin secretion. Therefore, we started an in vivo study on whether a glucose load was accompanied by enhanced plasma levels of ABA. We determined plasma ABA concentrations in healthy subjects during prolonged (240 minutes) oral or intravenous glucose tolerance tests (OGTT, IVGTT). In all subjects undergoing OGTT (n=10), plasmatic ABA concentrations increased 3-7 times over basal values, peaking at 60-120 min after glucose administration. A significant positive correlation was found between the ABA area under the curve (AUC) and the glucose AUC ($p < 0.05$). In 3 out of 5 IVGTT, little or no increase of ABA levels was observed. In the remaining two subjects, the ABA increase was similar to that during OGTT. Incretins may mediate the stronger stimulation of ABA release by oral glucose, since presence of GIP and GLP-1 potentiated the glucose-induced ABA release from an insulinoma cell line (by 8 and 3 times, respectively). In addition to pancreatic islets, we found that human adipose tissue (obtained from abdominoplasty) also releases ABA in response to high (25 mM) glucose (3-fold over controls). Accordingly, adipocytes obtained upon differentiation of either human mesenchymal stem cells or of 3T3-L1 cells, released ABA in the supernatant when exposed to high glucose concentrations (approximately 4-fold over control in both cell types).
At nanomolar concentrations, ABA stimulated glucose uptake (2-fold over untreated cells and in the same range of insulin-induced glucose uptake) in 3T3-L1 cells differentiated to adipocytes.
In conclusion, we provide the first evidence of a physiological rise of plasma ABA concentrations after a glucose overload in humans, which can contribute to enhanced glucose uptake by adipose tissues.

Effect of human DAAO variants on cellular D-serine concentration

Laura Caldinelli, Matteo Emilio Pagliani, Silvia Sacchi, Gianluca Molla and Loredano Pollegioni

The Protein Factory – Dip. Biotecnologie e Scienze Molecolari, Politecnico di Milano e Università degli Studi dell'Insubria, via Dunant 3, 21100 Varese, Italy; laura.caldinelli@uninsubria.it

Keywords: human DAAO variants, D-serine, schizophrenia

INTRODUCTION: In the human brain D-amino acid oxidase (hDAAO) degrades D-serine, a glutamate co-agonist necessary for the activation of the NMDA receptors: a reduced concentration of synaptic D-serine has been associated to schizophrenia susceptibility (1). The activity of hDAAO is known to be affected by the interaction with the protein pLG72 (2). In the database various SNPs in the hDAAO gene resulting in nonsynonymous substitutions are reported: the most known one resulted in the G331V replacement. Furthermore, a gene sequence carrying three nucleotide substitutions yielding the D31H and R279A replacements has been also deposited (3).
In order to evaluate the effect of these polymorphisms on the enzyme functionality, we expressed and characterized the corresponding hDAAO variants. The potential physiological role of these polymorphisms was then investigated measuring cellular D-serine concentration in stably transfected U87 human glioblastoma cells.

RESULTS: The D31H and R279A variants of hDAAO have been over-expressed in *E. coli* cells (~ 10 mg/L of broth), whereas the expression level of G331V variant as soluble protein was very low (< 0.1 mg/L). The K_m for D-serine of D31H and R279A variants was 2-fold higher with respect to the wild-type enzyme, whereas k_{cat} was similar. As compared to the wild-type enzyme, the D31H and R279A hDAAO variants did not show alterations in binding affinity for benzoate and chlorpromazine inhibitors, in the dimeric oligomerization state and in the interaction with pLG72, whereas the affinity for the FAD cofactor was higher (4- and 10-fold).
By using stably transfected U87 human glioblastoma cells, the EYFP-hDAAO-D31H and -R279A proteins were shown to correctly localize to peroxisomes and to produce a higher decrease in cellular D/(D+L) serine ratio than the wild-type hDAAO. On the other hand, the G331V variant did not localize to peroxisomes, formed aggregates and affected cell viability.
In conclusion, these results suggest that expression of D31H and R279A hDAAO variants could affect cellular D-serine concentration and its release at synapsis, and thus might be related to schizophrenia susceptibility.

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Study on the molecular basis of oxidative stress-related retinal pathologies.

Daniela Calzia [1], Silvia Ravera[1], Alessandro Morelli[1], and Isabella Panfoli [1].

[1]*Department of Biology, University of Genova, Genoa, Italy;*

INTRODUCTION Rods are associated with scotopic vision. Rod outer segment (OS), where phototransduction takes place [1], consists of a stack of flattened disks surrounded by the plasma membrane. Previous proteomic and biochemical analyses reported the functional expression of the respiratory chain complexes I–IV and F_1F_o-ATP synthase in OS disks. Our present study confirms the presence of an oxidative metabolism in rod OS, a more native sample than disks, isolated from bovine retinas [2]. Energy production and oxidative stress are believed to play a pivotal role in some retinal pathologies such as age-related macular degeneration (AMD) [3] and Retinitis pigmentosa (RP) [4]. Therefore, clarifying the ATP source in ROS could be fundamental for a better understanding of vision physiology, and of great help to the comprehension of pathology of common retinal diseases and of new targets for their treatment with antioxidant molecules.

RESULTS Rod OS were characterized for purity. An oxygen consumption (stimulated by glucose and reverted by rotenone, antimycin A and KCN) and a consistent ATP synthesis (0.560±0.084µmol/min/mg), sensitive to the ATP synthase inhibitors, was measured in OS. The presence of Cytochrome *c* oxydase (COX) and F_1F_o-ATP synthase in the sample was verified by Semiquantitative Western-immunoblotting.

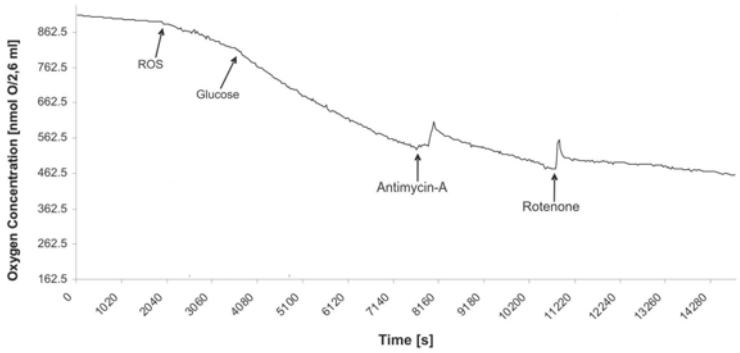

Fig.1 Oxygen consumption in Rod Outer segments after glucose addiction (0.2 mM), as respiratory substrate. Respiration rates decreased by addition of rotenone and antimycin A, respectively.

Morever COX and ATP synthase was catalytically active. COX and F_1F_o-ATP synthase with Rhodopsin co-localized in OS of mouse ocular sections, as found by immunohystochemical analysis. Data indicate that an oxidative phosphorylation occurs in rod OS, which do not contain mitochondria, thanks to the presence of ectopically located mitochondrial proteins.

This study may shed light on the pathogenesis of many retinal degenerative diseases that correlate with energy availability or oxidative stress and on the efficacy of empirical treatments, such as hyperbaric or vitamin therapies, in these pathologies.

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HIV-protease inhibitors exert anti-angiogenic and anti-tumor effects by inhibiting MMP-9 in a transgenic mouse model of cervical cancer

Stefania Capano[1], Federica Maione[1], Claudia Meda[1], PaoloMonini[2], Barbara Ensoli[2], Federico Bussolino[1] and Enrico Giraudo[1]

[1] Laboratory of Transgenic Mouse Models, Institute for Cancer Research and Treatment (IRCC), and Department of Oncological Sciences, University of Torino School of Medicine, Candiolo, Torino.
[2] National AIDS Center, Istituto Superiore di Sanità- Roma

Keywords: cervical cancer, HIV-protease inhibitors

INTRODUCTION: Cervical cancer is one of the most common malignancy worldwide. Recent studies have shown that HIV-protease inhibitors (HIV-PIs, figure 1), widely used in highly antiretroviral therapy, led to a reduced incidence and regression of HIV-associated tumors in infected patients. Evidences indicate that this antitumor effect is not only the result of drug-mediated HIV suppression and immune reconstitution, but also of direct anti-angiogenic and anti-tumor actions. [1, 2]. Herein we sought to evaluate whether HIV-PIs could impair events leading to cervical cancer employing a mouse model of spontaneous cervical cancer, K14-HPV/E_2.

RESULTS: We performed a prevention-intervention trial aimed to prevent or regress tumor progression and angiogenesis by treating mice with Indinavir, Saquinavir and Ritonavir. HIV-PIs indirectly inhibited tumor growth and incidence by exerting an antiangiogenic effect and, consequently, by inducing tumor apoptosis. We further performed a regression trial, starting treatments when the mice had invasive tumors, to evaluate if HIV-PIs were able to regress or stabilize tumor progression. Interestingly HIV-PIs dramatically reduced tumor burden and invasiveness by increasing collagen-IV expression compared to controls. Remarkably, in both treatments, HIV-PIs completely inhibited matrix metalloprotease (MMP)-9 activity in tumors compared to controls. Since MMP-9 is involved in the mobilization of VEGF-A from the matrix, we evaluated the formation of VEGF-A-VEGR-2 complex in tumor vessels. Of note HIV-PIs strongly reduced the amount of VEGF bound to its receptor VEGFR2, in parallel to a dramatic reduction in MMP-9 activity, suggesting that HIV-PIs, by targeting MMP-9, impair the VEGF-A pathway and therefore inhibit tumor angiogenesis.

We conclude that HIV-PIs have anti-angiogenic and anti-tumor effects on cervical carcinogenesis unrelated to their antiviral activity and that these drugs could represent new antiangiogenic targets that can be easily transferred to the clinic.

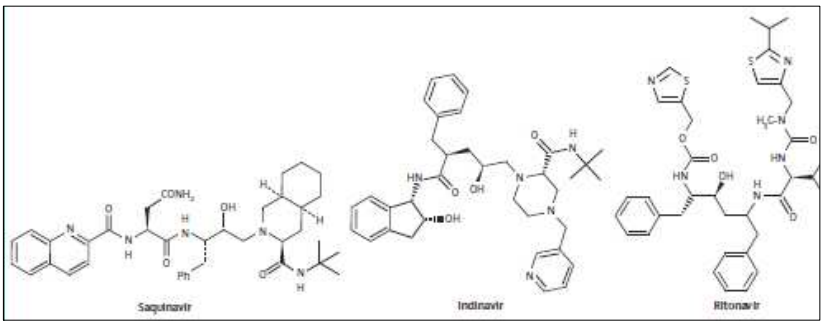

Figure 1- Chemical structure of approved protease inhibitors (3)

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Biochemical pathways of arsenic stress in *Pteris vittata* roots colonized by arbuscular mycorrhizal fungi.

Chiara Cattaneo, Elisa Bona, Patrizia Cesaro, Francesco Marsano, Graziella Berta and Maria Cavaletto

Dipartimento di Scienze dell'Ambiente e della Vita (DISAV), Università del Piemonte Orientale "A. Avogadro"- Alessandria, Novara, Vercelli Italy. chiara.cattaneo@mfn.unipmn.it

Keywords: arsenic, *Pteris vittata*, roots, proteome, arbuscular mycorrhizal fungi

INTRODUCTION: Arsenic is a toxic metalloid and its massive spread in the environment is mainly due to anthropogenic activities. Despite its phytotoxicity, some plant species can store high amounts of arsenic in their tissues, allowing the use of phytoextraction to remove As from contaminated areas. *Pteris vittata* has been the first arsenic hyperaccumulating plant to be discovered (1). This fern can tolerate very high arsenic concentration in soil and accumulates the metalloid in its fronds. However, its capability to tolerate arsenic has not been completely investigated. Arbuscular mycorrhizal (AM) symbiosis is a widespread mutualism in nature and various plant taxa, including ferns, benefit from it. AM fungi colonize plant roots, improving plant mineral nutrition and promoting plant tolerance/resistance to several stresses. It has been observed that plants growing on arsenic polluted soils are usually mycorrhizal and that AM fungi enhance arsenic tolerance in a number of plant species. The aim of the present work was to study the effects of the AM fungus *Funneliformis mosseae* (ex *Glomus mosseae*) on arsenic-treated *P. vittata* plants by the use of a proteomic approach.

RESULTS: AM symbiosis induced a significant decrease of arsenic content in roots. Image analysis showed that 37 spots were differently affected (21 identified by MS/MS analysis). Arsenic treatment affected the expression of 14 spots, the combination of arsenic and *F. mosseae* modulated 3 spots, while *F. mosseae* alone modulated 17 spots. Comparative analysis of arsenic- and mycorrhizal-induced proteins in *P. vittata* roots revealed that glycolytic enzymes were strongly involved. Moreover up regulation of aldehyde dehydrogenase represented a response to oxidative stress. In presence of *F. mosseae* colonization and arsenic, a decrease of S-adenosylmethionine synthase was detected, suggesting an alleviating effect for mycorrhization (2). To conclude, stress symptoms were observed after arsenic treatment even in the arsenic-hyperaccumulator *P. vittata*, while AM symbiosis sorted a protective effect toward arsenic stress.

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Neuroprotective role of fatty acid on diabetic neuropathy

Cermenati G.[*,#], Abbiati F.[#], Cermenati S.[$], Brioschi E.[*,#], Volonterio A.["], De Fabiani E.[#], Crestani M.[#], Garcia-Segura L.M.[§], Melcangi R.C.[°], Caruso D.[#], Mitro N.[*,#]

[*]Giovanni Armenise-Harvard Foundation Laboratory, Dept. of Pharmacological Sciences, Università degli Studi di Milano, Milano, Italy; gaia.cermenati@unimi.it.
[#]Laboratory of Biochemistry and Molecular Biology of Lipids and Mass Spectrometry "Giovanni Galli", Dept. of Pharmacological Sciences, Università degli Studi di Milano, Milano, Italy.
[°]Dept. of Endocrinology, Pathophysiology and Applied Biology - Center of Excellence on Neurodegenerative Diseases, Università degli Studi di Milano, Milano, Italy.
[$]Dept. of Biomolecular Sciences and Biotechnologies, Università degli Studi di Milano, Milano, Italy.
["]Dipartimento di Chimica, Materiali ed Ingegneria Chimica "Giulio Natta" Politecnico di Milano, Milano, Italy.
[§]Instituto Cajal, C.S.I.C., Madrid, Spain.

INTRODUCTION: Peripheral neuropathy (PN) is a frequent complication of diabetes mellitus[1]. Approximately 50% of patients with this pathology developed PN and in most cases this condition becomes irreversible[2,3]. Myelin is a biological membrane characterized by high lipid content and it is one of the main structures that contribute to a correct function of the nervous system. The particular characteristics of the lipids present in the sheath provide the electrically insulating property required for the saltatory propagation of the nervous influx[4]. Liver X Receptors (LXRs) are ligand activated transcription factors belonging to the nuclear receptors superfamily. LXRs activation induces expression of a battery of genes regulating cholesterol homeostasis and modulating lipid metabolism. LXRs directly regulate the expression of the lipogenic transcription factor Sterol Regulatory Element Binding Factor-1c (SREBF-1c), a gene involved in fatty acid synthesis. Recently it has been highlighted the role of SREBF-1c in the regulation of lipid metabolism during peripheral nerve myelination.

RESULTS: Using experimental model of diabetic neuropathy (streptozotocin (STZ)-rats) we have observed that in STZ-rats treated with vehicle and STZ-rats treated with GW3965 (a LXRs synthetic ligand) the body weight was decreased and glucose levels were increased compared with the non-diabetic control. We demonstrated that diabetes decreased thermal sensitivity and nerve conduction velocity in STZ-treated rats, parameters rescued by the treatment with the LXRs ligand. Moreover, we observed that treatment with LXRs ligand counteracts alterations in myelin caused by diabetes improving myelin lipid content and restoring the expression levels of all major enzymes involved in fatty acid generation. These results suggest that increased lipogenesis in sciatic nerve may protect peripheral nerves from diabetic neuropathy.

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Secondary accumulation of gangliosides in sphingolipidosis.

Elena Chiricozzi, Simona Prioni, Vanna Chigorno, Alessandro Prinetti and Sandro Sonnino.

Center of Excellence on Neurodegenerative Diseases; Department of Medical Chemistry, Biochemistry and Biotechnology; University of Milan; 20090 Segrate; Italy elena.chiricozzi@unimi.it

Key words: sphingolipids, sphingomyelin, glycosphingolipids, gangliosides, sphingolipid storage diseases, Niemann-Pick.

INTRODUCTION: Sphingolipid metabolism is deeply deregulated in several pathologies. This seems to be responsible of neurodegeneration, in sphingolipidosis. Here, we focus the attention on secondary alterations of sphingolipid metabolism that have been sporadically reported in the literature, in some sphingolipidosis.
We present a detailed analysis of the lipid composition in different tissues from the acid sphingomyelinase-deficient mouse (ASMKO), the animal model for Niemann-Pick disease type A, characterized by the accumulation of sphingomyelin (SM). The animal model of NPD type A, was developed using gene targeting and embryo transfer techniques.

RESULTS: Results show, together with a general accumulation of SM, an unexpected tissue specific selection of the accumulated molecular species of SM, and of GM3 and GM2 gangliosides, that cannot be solely explained by the lack of sphingomyelinase. We observed the preferential accumulation of SM molecular species with shorter acyl chains in the nervous system, but not in extraneural tissues. The unbalance toward C18/C16-fatty acid containing SM species was detectable as early as SM accumulation started, and monosialoganglioside accumulation followed immediately afterwards. These changes in sphingolipid patterns should thus represent the effect of secondary biochemical pathways altered as a consequence of a non-related primary cause. The mechanism underlying these changes still remains to be elucidated and is probably the result of changes in the expression and/or activity of more than one single enzyme, and/or of anomalies in the traffic of the substrate/product concentrations in multiple cellular compartments. Several pieces of evidence suggest that altered sphingolipid metabolism results in a non-physiological plasma membrane composition and organization, leading to altered plasma membrane-originated signalling pathways that could be relevant to the onset of cellular damage and of tissue pathology.

8-PRENYLNARINGENIN, A NEW PLAYER IN PLATELET INHIBITION

C. Di Vito[1], S. Sampietro[1], M. Nalin[1], A. Bertoni[1,2], F. Sinigaglia[1,2]

[1]Department of Clinical and Experimental Medicine, University of Eastern Piedmont "A. Avogadro", Novara, Italy.

[2]Center of Biotechnology for Applied Medical Research (BRMA), Novara, Italy.

Phytoestrogens are plant-derived polyphenolic compounds that mimic mammalian estrogens. It has been reported that a strong correlation between the flavonoid intake and a decrease risk of cardiovascular diseases exists. The flavanone 8-prenylnaringenin (8-PN), extracted from the lupulin glands of the hop flowers, was identified as a novel phytoestrogen, unique with respect to estrogen receptors specificity and potency. Human exposure to 8-PN occurs primarily through the consumption of the hop-derived products, such as beer and an increasing number of dietary supplements. However, no investigations on the 8-PN role in modulating platelet function has been undertaken. Given these considerations, we have evaluated whether 8-PN could influence platelet functionality.

Our results showed for the first time that 8-prenylnaringenin was able to irreversibly inhibit platelet aggregation induced by different agonists and platelet adhesion to collagen matrix. Moreover, our data demonstrated that 8-PN effect on platelet function was not related to its ability to bind the estrogen receptors. The treatment of platelets with 8-PN directly activated one of the best characterized inhibitory pathway in platelets increasing intracellular cAMP and cGMP levels and promoting VASP phosphorylation. However these molecular events were not responsible for the inhibitory action of 8-PN on platelets. Therefore we focused our attention on protein phosphorylation, crucial event in platelet activation. Our results demonstrated that 8-PN inhibited the collagen-induced protein tyrosine phosphorylation and the subsequent activation of Pyk2, Akt, and Erk 1/2.

In conclusion we have demonstrated that 8-PN exerts antiaggregatory and antiadhesive effects on human platelets, independently of estrogen receptors. However further studies are needed to better characterize the molecular mechanisms involved in 8-PN-mediated platelet inhibition.

Inhibition of class I histone deacetylases unveils a mitochondrial signature and enhances oxidative metabolism in skeletal muscle and adipose tissue

A. Ferrari, A. Galmozzi, N. Mitro, E. Gers, F. Gilardi, C. Godio, G. Cermenati, D. Caruso, *A. Mai, **E. Saez, E. De Fabiani, M. Crestani.

Dipartimento di Scienze Farmacologiche Università degli Studi di Milano, *Dipartimento Studi Farmaceutici Università degli Studi di Roma "La Sapienza", **Department of Chemical Physiology The Scripps Research Institute La Jolla CA

Many transcriptional and epigenetic mechanisms are involved in the regulation of lipid metabolism. Histone deacetylases and nuclear receptors play an important role in the regulation of lipid metabolism in adipose tissues, liver and skeletal muscles in normal and disease states. It has been showed that mitochondrial dysfunction associated to unbalanced energy intake and expenditure could be a possible cause to the onset of obesity and insulin resistance. By using selective biochemical inhibitors here we show that class I histone deacetylases (HDACs) are important regulators of mitochondrial function. MS275, a class I selective HDAC inhibitor, increased mitochondrial biogenesis and oxidative metabolism in C2C12 murine myotubes via upregulation of the coactivator PGC-1α, a key determinant of mitochondrial biogenesis. Knock down of HDAC3 by RNAi increased the expression of PGC-1α and recapitulated the effects of MS275. Administration of MS275 to *db*/*db* mice improved the obese and diabetic phenotype, by reducing body weight, fasting glucose and insulin and by increasing insulin sensitivity. Metabolic studies showed elevated oxygen consumption in mice on MS275 and the concomitant decrease of the respiratory exchange ratio suggested a switch to oxidative metabolism. In addition, higher heat production was noticed in mice treated with MS275, an effect paralleled by the improved functionality of brown adipose tissue consequent to increased expression of typical marker genes such as Ucp1, Prdm16, Adrb3, Pgc-1α. In white adipose tissue (WAT) the treatment with MS275 reduces adipocytes size and promotes lipid catabolism and mitochondrial biogenesis. Remarkably, visceral white adipose tissue (WAT) of mice treated with MS275 underwent a major reprogramming as several markers of brown fat were increased in a Prdm16-independent fashion. At the same time, infiltration of macrophages and the expression of inflammatory markers in WAT were reduced. In conclusion, biochemical inhibition of class I HDACs revealed a mitochondrial signature mediated by the transcriptional coactivator Pgc-1α in skeletal muscle and by the Pgc-1α/Pparγ axis in adipose tissue, leading to insulin sensitizing effect in *db*/*db* mice.
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Novel strategies to deliver melatonin for the control of prostate cancer growth

E. Finati, L. Terraneo, G. Demartini, L. De Angelis°, F. Fraschini*, M. Samara, R. Paroni

Dpt.of Medicine, Surgery and Dentistry, °Dpt. of Pharmacological Sciences, *Dpt. of Pharmacology, Chemotherapy and Medical Toxicology, University of Milan, Milan, Italy, elena.finati@unimi.it

Keywords: Melatonin, SLN, cryo-laser therapy, prostate cancer cells.

INTRODUCTION: Melatonin, a neurohormone synthesized in the pineal gland, mediates many endocrine and behavioral processes, and modulates malignant cell proliferation, via MT1 receptor activation or dihydrotestosterone-induced calcium influx attenuation (1,2). Melatonin has also important antioxidant and antiangiogenic properties, mediated by the inhibition of the hypoxia inducible factor (HIF)-1 alfa. Solid-lipid nanoparticles (SLN) is a technology for drug delivery to target the drug against a specific organ, with control of the pharmacokinetics and optimization of their uptake into cancer cells. The cryopass laser therapy is a procedure consisting in the topical application of a frozen drug emulsion followed by a laser scan of the area to give the energy to penetrate the cutaneous barrier and actively deliver the active principle to the target area (**Fig.1**).

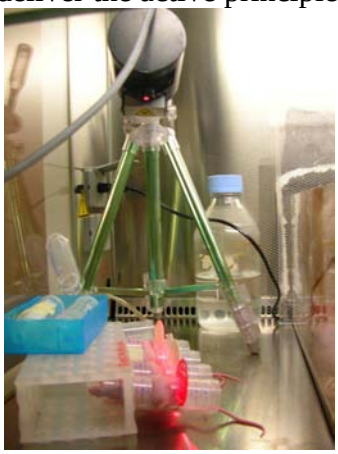

Fig.1

METHODS: We used an in-vivo model of nude mice xenograft of human LNCaP prostate cancer cells and compared the response of the xenografts to different routes of melatonin delivery: (a) i.p. as aqueous solution (1 mg/kg, n=7); (b) i.p. included in SLN (1 mg/kg, n=7) and (c) topical by cryolaser (4 mg/kg, n=11). Each group included also the respective control animals. The treatment schedule was the same for all the groups: 3 per week, extended for 6 week (17 treatments). Along the treatment period, the mice growth was recorded as well as the tumour volume. At the end (42^{th} day), the animals were sacrificed, the tumour collected, weighted, cut into 3 parts, one for histology, one for biochemistry, and the last for immunohistochemistry. Plasma and red blood cell were frozen at -80°C. Heart, brain, prostate, kidney, muscle, liver were collected and stored at -80 °C.

RESULTS: Both in controls and in treated animals the success of the xenografts was > 85% without significant differences. Tumour growth curves showed a similar trend in both i.p. melatonin treated groups (solution and SLN), but with a marked delay in respect to controls. The mean weight of the tumours collected 42 days after the xenograft was significantly lower in melatonin-treated mice respect to saline-treated controls (0.07±0.02 g *vs* 0.17±0.02 g, $p<0.05$). SLN-melatonin, by contrast, did not produce the same inhibitory effect on tumour growth (0.14±0.05 g), but histological analysis revealed a different tissue composition. Mice receiving topical melatonin with the laser scan, showed from the 27^{th} day of treatment, a significantly lower tumour growth kinetics in respect to the controls. At the sacrifice, the mean tumour weight of the laser-treated mice was lower than in controls one ($p<0.05$).

The results obtained by this investigation could be the basis for the future introduction of this natural molecule as adjuvant active component in therapeutic strategies for the treatment of malignant prostate cancer in humans, for the prevention of cancer relapses.

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Involvement of class I histone deacetylases in the regulation of Cholesterol 7α hydroxylase gene and on bile acid metabolism

Fiorino E.[*], Multineddu C.[*], Gilardi F.[*§], Giudici M.[*], Galmozzi A.[*], Mitro N.[*], De Fabiani E.[*], Caruso D.[*], Crestani M. [*]

*Lab "G. Galli", Biochim Biol Mol-Spet Massa, Dpt Pharmacological Sciences, University of Milan. erika.fiorino@unimi.it.
§ Center for Investigative Genomics, Genopode Building, University of Lousanne,

INTRODUCTION: Cholesterol 7α-hydroxylase (CYP7A1) catalyzes the hydroxylation of cholesterol at the 7α position, the major check-point in the classic pathway of bile acid (BA) biosynthesis. BA returning to liver via enterohepatic circulation repress *CYP7A1* expression. We previously demonstrated that BA induce the sequential recruitment of histone deacetylases (HDAC) 7, 3, and 1, of the corepressors Silencing Mediator of Retinoid and Thyroid receptors-α (SMRT-α) and N-CoR1 on *CYP7A1* gene promoter creating a complex that repress *CYP7A1* gene transcription. Moreover we defined that treatment with valproic acid and trichostatin A, two non-selective HADC inhibitors, totally prevent the repression exerted by BA on *CYP7A1* expression *in vitro* and *in vivo* consequently decreasing LDL-cholesterol in mice. Based on these results the aim of our study was to define the role of specific HDACs and corepressors in the regulatory mechanism of *CYP7A1* gene transcription and on bile acid metabolism. To this end we tested the pan inhibitor SAHA, the class 1 selective HDAC inhibitor MS275 and the class 2 selective HDAC inhibitor MC1568 *in vitro* and *in vivo*.

RESULTS: Our results show that the modulation of class 1 HDAC activity with the selective inhibitor MS275 prevents the negative feedback of BA on *CYP7A1* expression *in vitro* and *in vivo*. Moreover HDAC1 silencing with siRNA oligonucleotides in a hepatic reporter cell line with siRNA oligonucleotides increases *CYP7A1* transcription. In conclusion our data highlight the role of class 1 HDACs in the formation of the repressive complex that acts on *CYP7A1* promoter and suggest that the modulation of HDAC1 activity with a selective inhibitor may affect cholesterol and bile acid metabolism.

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Characterization of D-amino acid oxidase from rat: implications for the development of new drugs to treat schizophrenia

Luca Frattini, Loredano Pollegioni, Silvia Sacchi, Gianluca Molla and Luciano Piubelli

The Protein Factory – Dip. Biotecnologie e Scienze Molecolari, Politecnico di Milano e Università degli Studi dell'Insubria, via Dunant 3, 21100 Varese, Italy; luca.frattini@uninsubria.it

Keywords: Flavoprotein, Schizophrenia

INTRODUCTION: D-amino acid oxidase (DAAO) is a FAD-containing flavoprotein which dehydrogenates the D-isomer of amino acids to the corresponding α-keto acid and ammonia, reducing molecular oxygen to hydrogen peroxide. Human DAAO (hDAAO) in the brain plays a key role in the pathophysiology of schizophrenia by modulating the D-serine level (1). Rat is now widely used as in *vivo* model for screening and testing of new drugs for schizophrenia treatment based on DAAO inhibition (2), anyway a detailed biochemical and structural characterization of rat DAAO (rDAAO) is absent.

RESULTS: The rDAAO cDNA was synthesized based on the amino acid sequence deposited in data bank (3); it was then cloned in pET28b plasmid and rDAAO has been overexpressed in BL21(DE3) Star *E. coli* cells using the same conditions set up for hDAAO (4). Recombinant rDAAO was purified to 95% homogeneity by a single-step procedure on metal-chelate affinity column. A yield of 10 mg of protein/liter of culture was achieved.
rDAAO is a monomer both in the holo- and apoprotein forms, whereas hDAAO is a stable homodimer (1). The K_d for FAD binding to rDAAO apoprotein is 3.5×10^{-7} M, similar to that of DAAO from pig kidney and 20-fold lower than the value determined for hDAAO. The K_m for D-Ala and D-Ser are about 100- and 40-fold higher with respect to the human enzyme, whereas k_{cat} values are 5- and 2-fold higher. rDAAO shows the highest affinity for bulky and hydrophobic substrates, such as D-Trp and D-Phe. Furthermore, K_d for binding of classical DAAO inhibitors such as benzoate and anthranilate are 20- and 130-fold higher, respectively, than for binding to the human homologue.
To investigate the effect of rDAAO on D-serine level in cultured human cells, U87 human glioblastoma cells were transfected with the pEYFP-C3 plasmid (the rDAAO cDNA was inserted in-frame with the gene coding for EYFP), and stable clones were selected using G418. The cellular D- and L-serine levels were determined by HPLC analysis; the values were normalized to L-serine concentration by using D/(D+L) serine ratio. The results showed a D/(D+L) serine ratio in rDAAO-transfected U87 cells similar to the values observed in nontransfected cells and 4-fold higher with respect to U87 cells transfected with hDAAO.
In conclusion, rDAAO seems to differ significantly from the human enzyme concerning the properties related to substrate and inhibitor binding. Moreover, the presence of rDAAO in transfected human cell does not affect the D-serine cellular level. These results raise doubts on the use of rat as model system for testing new drugs against schizophrenia.

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SDSL-EPR as a tool for grasping information on the orientation of proteins adsorbed onto nanoparticles

Elena Ghibaudi*, Francesco Turci*‡, Massimiliano Colonna*‡, Barbara Boscolo*, Ivana Fenoglio*‡ and Bice Fubini*‡

*Dip. Chimica IFM; ‡Interdept. Center "Scansetti" for Studies on Asbestos and other Toxic Particulates, University of Torino, via Giuria 7, 10125 Torino, Italy; elena.ghibaudi@unito.it

Keywords: EPR spectroscopy, SDSL, protein corona, nanoparticles.

INTRODUCTION: A nanoparticle (NP) in contact with a biological medium is quickly covered by a dynamic layer of proteins found in the medium, known as "protein corona". The corona is established through a competitive process driven by thermodynamic and kinetic factors, such as the stability of the NP-protein adduct, the relative concentrations of proteins in the medium, the charge distribution on the NP as well as on the protein surface (which are both pH-dependent), the chemical nature and reactivity of the NP surface, the temperature, etc. [1]. As the cell "sees" protein-coated NPs rather than bare NPs, the chemical and topological characterization of the protein corona is essential to understand the mode of interaction of NPs with cells and to predict the fate of NPs inside cells. Adsorption of a protein onto a solid surface may induce structural changes that reflect on the protein behaviour, e.g. on enzyme activity and on molecular recognition processes that rely on a correct protein folding. Even if the general fold is not compromised, the orientation of the protein onto the surface may still influence its functionality as the entrance of the substrate channel may be hindered or the diffusion of substrates within the active site compromised. Site-directed spin-labeling (SDSL) coupled with EPR spectroscopy allows to grasp information on the orientation of proteins towards a surface as well as on their conformational state [2]. It relies on the conjugation of a paramagnetic probe at specific sites of the peptide chain (usually Cys residues that may be introduced by site-directed mutagenesis).

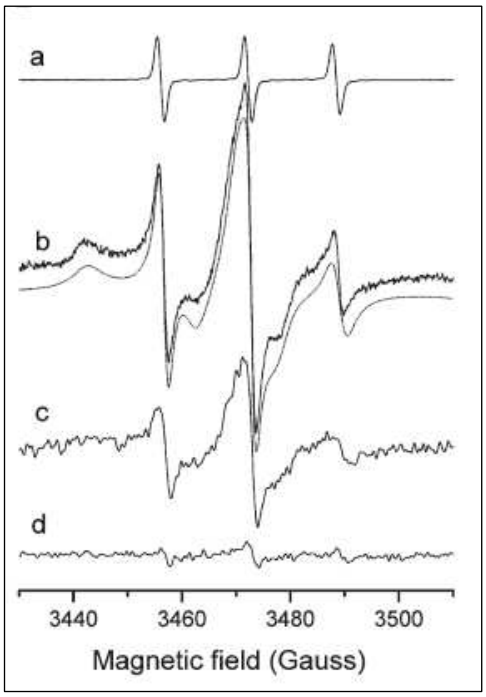

Fig. 1. EPR spectra of His19-labelled RNase on silica NPs [3]

RESULTS:We employed SDSL–EPR to investigate the interaction of lysozyme and ribonuclease with nanostructured silica particles [3]. We were able to show that both proteins expose preferably the labelled side to the surface, although their adsorption modes are different. In fact, the labelled domain of lysozyme adheres quite flatly to the silica surface, whereas RNase adsorption results in a probe-containing cavity that is almost inaccessible from the outside, although it still allows a certain degree of mobility of the spin-probe which is trapped inside. In both cases, some protein desorption occurred with time; in the case of RNase, some structural rearrangements of the adsorbed molecules were observed as well.

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Haemozoin-dependent pro-inflammatory molecules promote Tissue Inhibitor of Metalloproteinase-1 expression and release through activation of NF-kappaB pathway in human adherent monocytes

Giuliana Giribaldi, Daniela Ulliers, Elena Valente, Amina Khadjavi, Giorgia Mandili, Manuela Polimeni and Mauro Prato

Department of Genetics, Biology and Biochemistry, Medical School, University of Torino
Via Santena 5 bis, 10146 Torino Italy; mauro.prato@unito.it

Keywords: Haemozoin, malaria, Tissue Inhibitor of Metalloproteinase-1, Matrix Metalloproteinase-9, Monocyte Inflammatory Protein-1, Tumor Necrosis Factor alpha, Interleukin-1beta

INTRODUCTION: Growing evidence on the involvement of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) in clinical progress towards complicated malaria has been emerging in the last decade. A role for deregulated MMP/TIMP balances in blood-brain barrier (BBB) damage during cerebral malaria (CM) was suggested *in vivo* (1,2), whereas malarial pigment (HZ, haemozoin) was shown to enhance *in vitro* inflammation-dependent MMP-9 expression and release through activation of the NF-kappaB pathway in human monocytes (3-6). Moreover, high serum levels of TIMP-1, endogenous inhibitor of MMP-9, were found in patients with severe malaria (7). In the present study, the effects of HZ on TIMP-1 mRNA expression and protein release were studied in human monocytes, along with dependence on production of pro-inflammatory molecules and involvement of NF-kappaB activation.

RESULTS: HZ promoted TIMP-1 mRNA expression and protein release. As expected, HZ also enhanced Macrophage Inflammatory Protein-1alpha (MIP-1alpha), Tumor Necrosis Factor alpha (TNFalpha) and Interleukin-1beta (IL-1beta) mRNA expression and protein release. All the HZ effects on TIMP-1 were mimicked by recombinant (r) human (h) MIP-1alpha, rhTNFalpha and rhIL-1beta, while they were abrogated by anti-hMIP-1alpha, anti-TNFalpha and anti-IL-1beta antibodies. Quercetin, artemisinin and parthenolide, three NF-kappaB inhibitors previously shown to inhibit the HZ-enhanced release of MMP-9, TNFalpha and IL-1beta (5), abrogated the HZ effects on MIP-1alpha and TIMP-1 release.
In conclusion, the present data show that phagocytosis of HZ by human monocytes promotes an inflammation-mediated increase of expression and release of TIMP-1 through activation of the NF-kappaB pathway and fit with previous evidence suggesting a role for TIMP-1 in clinical diagnosis as a marker for severe malaria.

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SCREENING OF ABSCISIC ACID INHIBITORS IN HUMAN CELLS

Alessia Grozio, Enrico Millo, Lucrezia Guida, Mirko Magnone, Annalisa Salis,Tiziana Vigliarolo, Antonio De Flora and Elena Zocchi

Dep. of Experimental Medicine (DI.ME.S) and Center of Excellence for Biomedical Research, University of Genova, Italy, alessia.grozio@libero.it

Keywords: Abscisic Acid, Aptamer

INTRODUCTION: Abscisic acid (ABA) is a phytohormone involved in fundamental physiological processes in higher plants and in lower Metazoa (1-2). Recently we have demonstrated that ABA stimulates several pro-inflammatory activities [phagocytosis, reactive oxygen species and nitric oxide (NO) production, and chemotaxis] of human granulocytes through cAMP and cADPR (3). In human monocytes ABA evokes an intracellular Ca^{2++}rise through the second messenger cyclic ADP-ribose, leading to NF-κB activation and consequent increase of cyclooxygenase-2 expression and prostaglandin E2 (PGE2) production and enhanced release of MCP-1 (monocyte chemoattractant protein-1) and of metalloprotease-9, all events reportedly involved in atherogenesis (4). Twenty-two analogs of ABA, natural or synthesized in our laboratory were tested for ABA antagonistic effects on human granulocytes. Cells were incubated in the presence of ABA and of the various analogs and the concentration of cAMP was measured. Recently, we have selected by SELEX method (Systematic Evolution of Ligands by Exponential Enrichment)(5), sequences of ssDNA (aptamers) of 76 nucleotides from a library of random sequences, capable of selectively binding ABA.

RESULTS: A synthetic compound (N #10) capable of abrogating ABA-induced cAMP production by granulocytes was identified. Competition binding experiments showed that #10 has an IC50 of 0.87 nM and a Ki of 0.46 nM. Furthermore, at concentrations ranging between 1 nM and 1 μM, #10 significantly inhibited the ABA-induced functional activation of granulocytes. #10 (1 μM) also abrogated monocyte production of PGE_2 and MCP-1 induced by ABA. Stability of #10 in deionized water, phosphate buffer and in human plasma was verified by HPLC-MS and exceeded 90% after 24 hours at 37°C. Finally, #10 was not cytotoxic on several human and murine cell types. Besides, out of 22 clones selected, three ABA-binding sequences were identified by ELONA (Enzyme-Linked Oligonucleotide Assay). The K_D of these aptamers is approximately 1.5 μM and they are able to discriminate between the different ABA isomers and enantiomers.

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HPR resistance of a human ovarian carcinoma cell line is mediated by sphingosine kinase activity.

Giuditta Illuzzi[1], Caterina Bernacchioni[2], Massimo Aureli[1], Simona Prioni[1], Chiara Donati[2], Vanna Chigorno[1], Paola Bruni[2], Sandro Sonnino[1], and Alessandro Prinetti[1]

[1] Center of Excellence on Neurodegenerative Diseases; Department of Medical Chemistry, Biochemistry and Biotechnology; University of Milan; 20090 Segrate; Italy, giuditta.illuzzi@unimi.it
[2] Department of Biochemical Sciences, University of Florence, 50134 Florence Italy;

Keywords: Sphingosine kinase, human ovarian cancer cell line, drug resistance

INTRODUCTION: HPR is a preventive chemotherapeutic drug that induces ceramide production, a proapoptotic sphingolipid (1), in the A2780 cells. The A2780/HPR is a clonal cell line derived from A2780 human ovarian carcinoma cell line that is resistant to this cytotoxic effect. In literature are described alterations in the sphingolipid metabolism, increased glycosilation of ceramide by overexpression of the glucosilceramide synthase, that permits to scavange the apoptotic ceramide production (2). The principal catabolic pathway of ceramide leads to the production of sphingosine, that can be phosphorylated by the Sphingosine Kinase (SK) producing the sphingosine-1-phosphate (S1P), which is also a bioactive sphingolipid but with prosurvival and progrowth effects. S1P and ceramide are finely regulated in the cell by a particular mechanism,the rheostat, in which it is the ratio between the two cell lipids content, and not the absolute content of each, that determines the cell fate.

RESULTS: The production of S1P is significantly higher in A2780/HPR vs. A2780 cells and the analysis of SK activity resulted higher in A2780/HPR cells vs. A2780, due to the overexpression at mRNA and protein levels of the SK-1 isoform. The treatment with a potent and highly selective pharmacological sphingosine kinase inhibitor effectively reduced S1P production and resulted in a marked reduction of cell proliferation of A2780 and A2780/HPR cells.
Moreover the SK inhibitor sensitizes A2780/HPR cells to the cytotoxic effect of HPR, due to the alteration of intracellular S1P and ceramide levels. On the other hand, the overexpression of SK-1 in A2780 cells was sufficient to induce HPR resistance in these cells.
The role of SK in HPR resistance is not mediated by the S1P receptors, as suggested by the absence of effects after treatment of A2780 and A2780/HPR cells with agonists and antagonists of S1P receptors on the sensitivity to the drug.
These data clearly demonstrate a role for SK in determining resistance to HPR in ovarian carcinoma cells, due to its effect in the regulation of intracellular ceramide/S1P ratio which is critical in the control of cell death and proliferation.

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Protein nitration and nitric oxide signaling pathways during metamorphosis in *Ciona intestinalis*.

Elisa Maffioli[1], Armando Negri[1] , Elena Ercolesi[2], Gabriella Fiore[2], , Marco d'Ischia[3], Anna Palumbo[2], Gabriella Tedeschi[1]

[2]Cellular and Developmental Biology Laboratory, Stazione Zoologica Anton Dohrn, Villa Comunale, 80121 Naples, Italy
[1]D.I.P.A.V.-Section of Biochemistry, University of Milan, Via Celoria 10, 20100 Milan, Italy
[3]Department of Organic Chemistry and Biochemistry, University of Naples Federico II, Via Cinthia, 80126 Naples, Italy

Keywords: *Ciona intestinalis*, metamorphosis, nitic oxide

INTRODUCTION: Nitric oxide (NO) has recently been shown to play an important role in many biological function such as development of some marine invertebrates. In *Ciona intestinalis* NO is involved at later stages of development (1). This ascidian represents a good system of studies and its life cycle is characterized by a rapid development The process involves a regression of the tail controlled by caspase-dependent apoptosis. A recent study reports that NO regulates tail regression in a dose-dependent manner, acting on caspase-dependent apoptosis. A variation of NO levels result in a delay or acceleration of tail resorption. We provide now evidence that NO regulates *Ciona* metamorphosis via a complex balance of signaling pathways that are critically dependent on local redox control and temporal changes of reactive oxygen species production**.**

RESULTS: Protein nitration patterns were examined during larval development and metamorphosis by Western blot analysis of protein extracts at different developmental stages using an antibody against nitrotyrosine. These analysis reveal a variety of nitrated proteins from the first embryonic stage up to post-hatching and metamorphosing stages. In particular, increased protein nitration was observed at the late larvae stage, when larvae are competent, and proteomic methodologies identified nitrated tyrosine residues in ERK and snail. Moreover, exposure of larvae to NO resulted in a marked decrease in ERK phosphorylation.
In conclusion our results provide an important link between NO signalling and apoptosis during metamorphosis in *C. intestinalis*.

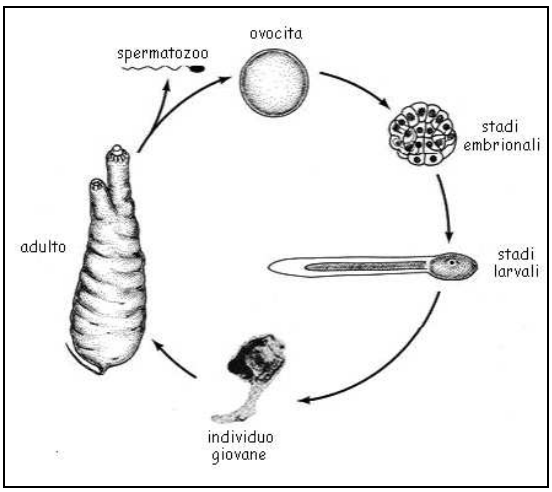

Fig.1 Life cycle of *C. intestinalis*

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Autocrine abscisic acid plays a key role in quartz-induced macrophage activation

Mirko Magnone[1], Laura Sturla[1], Emanuela Jacchetti[2], Sonia Scarfì[1], Santina Bruzzone[1], Cesare Usai[2], Lucrezia Guida[1], Annalisa Salis[1], Antonio De Flora[1] and Elena Zocchi[1]

[1] Department of Experimental Medicine (DIMES), Section of Biochemistry, and Centre of Excellence for Biomedical Research (CEBR), University of Genova; [2] Institute of Biophysics, Consiglio Nazionale delle Ricerche, Genoa, Italy.

INTRODUCTION: Inhalation of quartz induces silicosis, a lung disease where alveolar macrophages release inflammatory mediators including prostaglandin-E_2 (PGE_2) and tumor necrosis factor-α (TNF- α) leading to tissue damage (1, 2). The phytohormone abscisic acid (ABA) is a recently discovered human hormone involved in the activation of inflammatory cells (3, 4). Here we reported the pivotal role of ABA in silica-induced activation of murine RAW264.7 macrophages and of rat alveolar macrophages (AM).

RESULTS: Stimulation of both RAW264.7 and AM with quartz induced a significant ABA release (5-and 10-fold, respectively), compared to untreated cells. In RAW264.7, autocrine ABA released after quartz stimulation sequentially activated the plasmamembrane receptor LANCL2 and NADPH oxidase, generating a Ca^{2+} influx resulting in NF-κB nuclear translocation, PGE_2 and TNF-α release (3-, 2- and 3.5-fold increase, respectively) compared to control, unstimulated cells. Quartz-stimulated RAW264.7 silenced for LANCL2 or pre-incubated with a monoclonal antibody against ABA, showed an almost complete inhibition of NF-κB nuclear translocation, PGE_2 and TNF-α release (78%, 71% and 82%, respectively), compared to controls. These included RAW264.7 elecroporated with a scramble oligonucleotide or pre-incubated with an unrelated antibody. AM showed similar early and late ABA-induced responses as RAW264.7.
These findings identify ABA and LANCL2 as key mediators in quartz-induced inflammation providing possible new targets for anti-silicotic therapy.

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Uptake of nanoparticle-conjugated allergens by human monocytes

Mauro Marengo, Matteo Miriani, Francesco Bonomi, Stefania Iametti, Hanne Frøkiær*

Dipartimento di Scienze Molecolari Agroalimentari, Università degli Studi di Milano, Milano, Italy; *Department of Basic Sciences and Environment, University of Copenhagen, Copenhagen, Denmark

Keywords: allergens, nanobiotech

INTRODUCTION: Stable biocompatible magnetic nanoparticles, conveniently grafted with affinity ligands and/or transfection agents to integrate functionally and efficiently into cellular and subcellular structures, can confer cells magnetic activity, and may represent versatile and non-invasive tools for targeting specific cellular structures, and for monitoring cellular functions both in vitro and in vivo (1, 2).

RESULTS: In this study, betalactoglobulin, the most abundant protein in bovine whey and a major food allergens, was covalently conjugated to biocompatible carboxymethyldextran-coated magnetic nanoparticles. The conjugated protein retained its immunoreactivity towards different monoclonal antibodies. BLG-conjugated nanoparticles were taken up by human monocytes much more efficiently than non-conjugated particles, allowing easy magnetic separation of cells that had integrated the allergen. To assess whether the interaction between monocytes and the nanoparticles was associated to their internalization rather than to mere adsorption of the particles on the cell surface, BLG was conjugated to fluorescent-labeled magnetic nanoparticles. The uptake of these materials by human monocytes was monitored through flow cytometry and confocal microscopy, in comparison with fluorescent nanoparticles devoid of the allergen or conjugated with human serum albumin.
Both approaches confirm a higher uptake of the BLG-conjugated particles, and confocal microscopy provided clear evidence of particles internalization into the cytoplasm (Fig. 1). These results pave the way to use a combination of these approaches to improve the current understanding of the intracellular and intratissutal path of allergens that may be involved in the immune response to food.

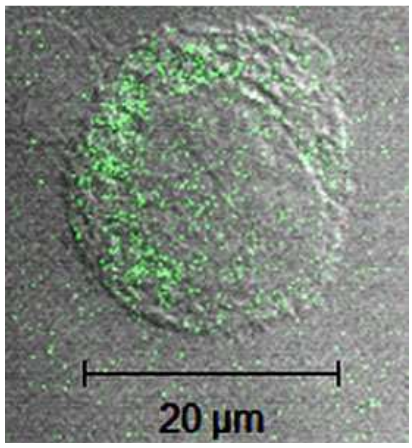

Fig. 1. Confocal microscopy evidence of uptake of BLG-conjugated fluorescent nanoparticles by human monocytes

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Hybrid gangliosides for the detection of anti-ganglioside complex antibodies.

Laura Mauri, Riccardo Casellato, Maria G. Ciampa, Ken-ichi Kaida,[1] Mayumi Motoyama,[2] Susumu Kusunoki,[3] and Sandro Sonnino

Department of Medical Chemistry, Biochemistry and Biotechnology, University of Milan, Via F.lli Cervi 93, 20090 Segrate, Italy; laura.mauri@unimi.it
[1] Division of Neurology, National Defense Medical College, Saitama, Japan,
[2] Department of Neurology, Kinki University School of Medicine, Osaka, Japan

Keywords: anti-oligosaccharide antibodies, ganglioside dimer, GBS

INTRODUCTION: Gangliosides are glycosphingolipids that are concentrated in the outer leaflet of neuronal membranes with exposure of their oligosaccharides on the cell surface (1). Guillain-Barre´ syndrome (GBS) is acute autoimmune neuropathy, often subsequent to an infection. Antiganglioside antibodies are often closely associated with clinical phenotype and specific symptoms of GBS. Recent studies demonstrated that some GBS patients had serum antibodies that specifically recognize the novel glycoepitopes formed by two individual ganglioside molecules and named such antibodies as 'anti-ganglioside complex (GSC) antibodies'. Those antibodies can be used as diagnostic markers of GBS. Conventional measurement of antiganglioside antibodies has been done for purified single ganglioside antigens using enzyme-linked immunosorbent assays (ELISAs) or thin-layer chromatogram (TLC)-immunostaining (2-4).

RESULTS: The availability of several hybrid gangliosides, containing two or more oligosaccharide chains and the availability of simple analytical approaches opens new perspectives for the understanding and therapy of several neuropathies. For this purpose we prepared the dimeric hybrid GM1-GD1a ganglioside derivative that contains two structural different oligosaccharide chains. A mixture of the two natural gangliosides was described to be recognized by the sera from patients with specific and clinical characterized GBS. We prepared the GM1-GM1 and GD1a-GD1a dimers to be used as controls. After removal of the acyls and reconstitution of the original acetylated sugars, the lyso-gangliosides, were connected with adipic acid to form the hybrid compound, a same-how mimic of the heterogeneous plasma membrane cluster of gangliosides. The dimeric hybrid GM1-GD1a was very well recognized by the GBS serum. However no reactivity was observed with the patient serum and the dimeric GM1 and dimeric GD1a. This suggests that both the GM1 and GD1a chain are necessary for a strong interaction and to maintain stable the antibody-antigen complex.

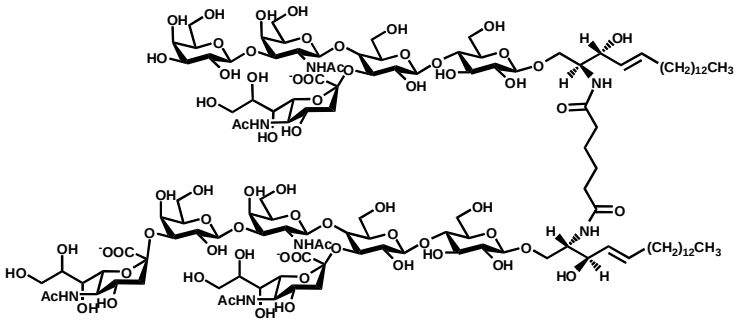

Fig. 1 Structure of GM1-GD1a hybrid ganglioside

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Cloning and expression of an artificial operon encoding a mini cellulase system in *Lactococcus lactis*

Silvia Piovano, Loredana Tarraran, Carlo Giunta, Enrica Pessione, Roberto Mazzoli

Dipartimento di Biologia Animale e dell'Uomo (DBAU), University of Torino, Via Accademia Albertina 13, 10123, Torino, Italy; sil1986@hotmail.it

Keywords: metabolic engineering, cellulosic biomass bioconversion

INTRODUCTION: Cellulose biomass is the most abundant waste produced by human activities including agricultural by-products (*e.g.* wheat straw and corn stalks), municipal solid wastes (*e.g.* waste paper) and industrial waste streams (*e.g.* paper mill sludge): it is definitely the most interesting substrate for the "biorefinery strategy" producing high-value products (chemicals, fuels, enzymes, etc..) by microbial fermentation. Lactic acid (LA) is among the most requested chemicals that can be produced by this approach, owing to the promising applications of its polymer, the polylactide, as a biodegradable and biocompatible general purpose plastic (*e.g.* for manufacturing of biomedical devices and for food and good packaging) alternative to petrochemical-derived materials. Lactic acid bacteria (LABs) are among the chief sources of LA: since natural LABs are not able to degrade cellulose. the present project aimed to construct a cellulolytic LAB by metabolic engineering so as to obtain a strain suitable for cost efficient consolidated bio-processings converting cellulosic wastes into optically pure LA.

RESULTS: The genes encoding two essential proteins, the cellooligosaccharidase BglA and the endoglucanase D (EngD) of the enzyme system involved in cellulose degradation in *Clostridium cellulovorans*, were cloned into *Lactococcus lactis*. Furthermore, the letter genes were assembled into an artificial bicistronic operon. *L. lactis* cells harbouring the gene engD showed endoglucanase activity, consistent with the biosynthesis of a catalitically active EngD. Further analyses, showed that, in the recombinant *L. lactis* strain, EngD is efficiently secreted in the extracellular medium. Current tests aim to asses if such recombinant *L. lactis* is able to efficiently grow on cellulose and its LA production yields

Optimizing HIV-1 protease production in *Escherichia coli* as fusion protein

Federica Volontè, Luciano Piubelli and Loredano Pollegioni

The Protein Factory – Politecnico di Milano and Università degli Studi dell'Insubria, via Dunant 3, 21100 Varese, Italy; luciano.piubelli@uninsubria.it

Keywords: HIV protease, protein overexpression

INTRODUCTION: Human immunodeficiency virus (HIV) is the etiological agent in acquired immunodeficiency syndrome (AIDS) and related diseases. The aspartyl protease encoded by HIV (HIV-1Pr) plays an essential role in viral maturation and is considered an attractive target for therapeutic intervention in the treatment of AIDS. In order to produce fairly large amounts of HIV-1Pr, in past years this protein was expressed as heterologous protein in *E. coli* by using different strategies. Anyway, the cytotoxicity (and low solubility) limited its overexpression: in most cases HIV-1Pr expressed in *E. coli* was recovered by refolding of inclusion bodies and/or the expression level was so low that it could be detected only by immunoblotting.
The purpose of this study was to produce an efficient and reproducible HIV-1Pr expression system and to establish a convenient (simple and fast) purification procedure of the recombinant active protein. The final aim is to employ recombinant active HIV-1Pr in the identification of new inhibitors, *e.g*. of the folding process.

RESULTS: A synthetic HIV-1Pr cDNA optimized for *E. coli* expression was cloned into different expression vectors that allow its production as fusion protein with bacterial periplasmic protein dithiol oxidase (DsbA) or with glutathione S-transferase (GST), also containing a six-histidine tag sequence. The protein expression was optimized by screening six different *E. coli* strains and five growth media: the best yield was achieved using *E. coli* BL21-Codon Plus(DE3)-RIL host grown in TB or M9 medium added of 1% (w/v) glucose. Among the different parameters assayed, the presence of a buffer system (based on phosphate salts) and a temperature of growth of 37 °C after IPTG addition played a major role in enhancing protease expression (up to 8 mg of DsbA:HIV-1Pr/L fermentation broth). For both DsbA:HIV-1Pr and GST:HIV-1Pr chimeric proteins, the overexpressed protein largely accumulated as unprocessed fusion protein in inclusion bodies. A simple refolding procedure on HiTrap Chelating column was developed which yielded a refolded, pure fusion protein with a > 80% recovery. Finally, enterokinase digestion of DsbA:HIV-1Pr gave more than 2 mg of HIV-1Pr per liter of fermentation broth but with a ≤ 80% purity, while PreScission protease cleavage of GST:HIV-1Pr yielded ~ 0.15 mg of pure HIV-1Pr per liter. Both enzyme preparations were fully active and stable (1).
This optimized expression and purification procedure represents a suitable way to produce fairly large amounts of high quality HIV-1Pr recombinant enzyme to be used in inhibitors development and characterization.

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Modulation of doxorubicin resistance by the glucose 6-phosphate dehydrogenase activity

[*]Manuela Polimeni, Claudia Voena, Joanna Kopekca, Chiara Riganti, Gianpiero Pescarmona, Amalia Bosia and Dario Ghigo

[*]Department of Genetics, Biology and Biochemistry, Via Santena 5/bis, 10126, Torino, Italy; manuela.polimeni@unito.it

Keywords: multidrug resistance, doxorubicin, glucose 6-phosphate dehydrogenase, pentose phosphate pathway, glutathione

INTRODUCTION: Tumor cells generally exhibit a cross-resistance to a large number of compounds chemically and structurally unrelated (multidrug resistance, MDR), partly determined by the overexpression of efflux pumps, such as P-glycoprotein and multidrug resistance-associated proteins (MRPs) (1). Some MRPs are involved in the co-transport of glutathione (GSH) that is required for the MRP-mediated extrusion of chemoterapeutic drugs (2). Furthermore, both carcinogenesis and MDR are frequently associated with increased oxidative stress and activation of the cellular redox metabolism (3). How anti-neoplastic agents induce MDR in cancer cells and the role of GSH in the activation of pumps such as the MRPs are still open questions. GSH is the most abundant cellular thiol and plays a pivotal role in cancer and MDR development (4). The pentose phosphate pathway (PPP) is the most important pathway for cellular GSH recycling because glucose 6-phosphate dehydrogenase (G6PD), the first and rate-limiting enzyme, produces the NADPH used by glutathione reductase to reduce glutathione disulfide (GSSG) to GSH.

RESULTS: We report that a doxorubicin-resistant human colon cancer cell line (HT29-DX), exhibiting decreased doxorubicin accumulation and increased intracellular GSH content and MRP1 and MRP2 expression in comparison to doxorubicin-sensitive HT29 cells, shows increased activity of the PPP and of G6PD. We observed the onset of MDR in HT29 cells overexpressing G6PD, accompanied by an increase of GSH; the G6PD inhibitors dehydroepiandrosterone (DHEA) and 6-aminonicotinamide (6-AN) reversed the increase of G6PD and GSH and inhibited MDR both in HT29-DX cells and in HT29 cells overexpressing G6PD. The activation of the PPP and an increased activity of G6PD could be necessary to some MDR cells to keep high the GSH content, which is in turn necessary to extrude anticancer drugs out of the cell. Our data could provide a new further mechanism for GSH increase and its effects on MDR acquisition.

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Ectopic Aerobic ATP Production on C6 Glioma Cell Plasma Membrane

Silvia Ravera[1], Maria Grazia Aluigi[1], Daniela Calzia[1], Paola Ramoino[2], Alessandro Morelli[1] and Isabella Panfoli[1]

1: Biology Dep.t, University of Genova, V.le Benedetto XV, 3, 16132 Genova Italy; silvia.ravera@gmail.com
2: DIPTERIS, Biology Dep.t, University of Genova, C.so Europa 26, 16132 Genova Italy

INTRODUCTION:
Brain tumors are responsible for approximately 2% of all cancer deaths. Gliomas, and glioblasotmas are the most common form of primary tumors of the central nervous system (CNS) and despite many therapeutic strategies, such as surgery and chemotherapy, survival rate for patients with malignant gliomas remains low. Extracellular ATP and other nucleotides and nucleosides were shown to play a pivotal role as signaling molecules in physiological and pathological conditions in the CNS. In fact, in several glioma cell lines, representative models of extremely aggressive cancer cells (1) ATP is a positive factor for one or more characteristics important for the abnormal growth and survival of these cells, including proliferation, survival (blockage of apoptosis), angiogenesis, and invasion. For example, ATP is a mitotic factor for glioma cells (2) and modulates cell proliferation in primarily cultured astrocytes of neonatal rat cortices and rat striatum.
An ectopic location of Fo F1-ATP synthase in many mammalian cell membranes has been reviewed (3) even though most authors ascribe to it functions other than ATP synthesis. Recently, we have reported an extra-mitochondrial ATP synthesis, demonstrating that the ectopic electron transfer chain (ETC) proteins were functional and could play an energetic role in isolated myelin vesicles (4,5)
Aiming at studying the source of extracellular ATP in glioma cells, by immunofluorescence and biochemical analyses, we have researched an extra-mitochondrial aerobic metabolism on the plasma membrane of rat C6 glioma cell line.

RESULTS(6): An ATP synthesis coupled to oxygen consumption was observed in plasma membrane isolated from C6 cells, sensitive to common inhibitors of respiratory chain complexes, suggesting the involvement of a putative surface ATP synthase complex. Immunofluorescence imaging showed that Cytochrome c oxydase colocalized with WGA, a typical plasma membrane protein, on the plasma membrane of glioma cells. Cytochrome c oxydase staining pattern appeared punctuate, suggesting the intriguing possibility that the redox chains may be expressed in discrete sites on C6 glioma cell membrane. Data suggest that the whole respiratory chain is localized on C6 glioma cell surface. Moreover, when resveratrol, an ATP synthase inhibitor, was added to culture medium, a cytostatic effect was observed, suggesting a correlation among the ectopic ATP synthesis and the tumor growth.
So, a potential direction for the design of new targets for future therapies may arise.

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Diacylglycerol kinase alpha: a new regolator of megakaryopoiesis and platelet function

Sampietro S.[1], Di Vito C.[1], Nalin M.[1], Zhong X.P. [3], Graziani A.[1,2], Sinigaglia, F[1,2]. Bertoni A.[1,2]
[1] Dept. Clinical and Experimental Medicine, University A. Avogadro, Novara, Italy. [2] BRMA, University A. Avogadro, Novara, Italy, [3] Division of Pediatric Allergy & Immunology, Duke University Medical Center, Durham, NC 27710
E-mail: sara.sampietro@med.unipmn.it

Diacylglycerol kinases (DGKs) phosphorylate diacylglycerol (DG) to phosphatidic acid (PA) thereby regulating DG- and PA-mediated signaling, respectively.
Megakaryocytes (MKs) differentiate from hematopoietic stem cells (HSCs) and give rise to proplatelets and platelets, small anucleated cell fragments which play a central role in hemostasis and thrombosis. Regulation of DGKs may modulate the availability of DG, thus regulating the activation of specific signaling pathways. However very little is known about the regulation of expression and activity of DGK isoforms in HSCs, MKs and platelets.
In order to investigate the involvement of DGK-alpha (DGKa) in megakaryopoiesis we compared the MK potential of murine bone marrow-derived HSCs purified from DGKa KO and WT mice. HSCs from DGKa KO were able to generate in culture 1.5-fold more mature MKs than WT cells. Moreover the number of polyploid cells (ploidy > 8N) was increased by 2.2-fold and the expression of CD41, a marker of platelet differentiation, was upregulated by 2-fold.
Moreover, to evaluate the role of DGKa in platelet formation we assessed platelet recovery following immune-induced thrombocytopenia. Even though WT and DGKa KO mice normally have comparable platelet count, platelet recovery was accelerated in DGKa KO mice, and remained higher up to 7 days.
Finally, in platelet function, the second messenger DG is involved in multiple signal transduction pathways that culminate in platelet aggregation. Therefore we sought to study platelet aggregation in WT and DGKa KO mice. Our preliminary data indicate that platelets from DGKa KO mice are more responsive to stimulation with thrombin, collagen and U46619 when compared to WT animals. These results suggest that DGKa, by modulating the availability of DG, negatively regulates megakaryopoiesis and platelet formation, as well as platelet function.

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Expression of human proline oxidase as recombinant protein

Elena Tallarita, Loredano Pollegioni, Stefano Servi, Gianluca Molla

"The Protein Factory" – Università degli studi dell'Insubria and Politecnico di Milano. elena.tallarita@uninsubria.it; elena.tallarita@mail.polimi.it.

Keywords: proline oxidase; recombinant protein expression

INTRODUCTION: Schizophrenia is a common, debilitating psychiatric disorder. Current models of schizophrenia suggest that this pathology is related to abnormalities of the glutamatergic neurotransmission; accordingly genetic studies have identified a number of genes (e.g., *PRODH1*) whose coded protein products are related to schizophrenia susceptibility through the modulation of the glutamatergic neurotransmission (1,2). *PRODH1* encodes for the mitochondrial flavoenzyme proline oxidase (PO): it catalyzes the first step of oxidation of proline to 1-pyrroline-5-carboxylate (P5C), a key metabolite that can be converted to glutamate. The *PRODH1* gene is an hot spot for mutations: 16 missense mutations have been identified, 10 of which are present at polymorphic frequencies and result in mild to severe effects on PO activity (3). Unfortunately, the correlation at the molecular level between PO activity and neurotransmission is still unclear.
Functional and structural *in vitro* characterization of recombinant human PO is the pre-requisite to shed light on the role of this enzyme in the functionality of glutamatergic synapse under both physiological and pathological conditions. With this aim we undertook the expression in *E. coli* of the full length PO and of several deletion variants.

RESULTS: The synthetic cDNA encoding for wild-type PO was designed to have a codon usage optimized for *E. coli* expression. Although various plasmids, *E. coli* strains and growth media were tested, no expression of full length PO was obtained. Therefore, several deletion variants of PO (lacking a protein region potentially problematic for expression) were designed based on a bioinformatic analysis. All PO deletion variants were produced by PCR and subcloned into the pET24 or pT7.7 expression vectors producing a C-terminal or N-terminal 6xHis-tag fusion protein, respectively.
The PO-BarrelN_His variant (encompassing the whole C-terminal catalytic domain residues from 176 to 570) was successfully expressed as soluble dimeric holoprotein up to 10 mg/liter of fermentation broth. The purified PO variant shows the typical spectral characteristics of FAD-containing flavo-proteins (absorbance maxima at 450 nm and 380 nm). Its specific activity on the physiological substrate L-proline is quite low: 20 mU/mg.
The expression of an active PO variant in *E. coli* paves the way for the production of additional deletion variants of PO, as well as of variants related to schizophrenia susceptibility. This will allow to clarify the effect of the reported *PRODH1* polymorphisms on the catalytic properties of the enzyme and on the L-proline concentration in selected cell lines.

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Engineering *Lactococcus lactis* with *C. cellulovorans* designer cellulosomes for cellulose biomass bioconversion

Loredana Tarraran, Silvia Piovano, Carlo Giunta, Enrica Pessione, Roberto Mazzoli

Dipartimento di Biologia Animale e dell'Uomo (DBAU), University of Torino, Via Accademia Albertina 13, 10123, Torino, Italy; loredana.tarraran@gmail.com

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INTRODUCTION: Cellulosomes are the most efficient protein systems involved cellulose biomass degration in nature. They are the high molecular weight multi-enzyme complex, organized by non-enzymatic scaffolding protein. Such scaffolding are multi-domain proteins able to bind the enzymatic subunits *via* the cohesin modules, and to anchor the whole complex to the cell surface through hydrophilic (SLH) domains. "Designer cellulosomes" have been proposed as a tool for both understanding cellulosome action and for biotechnological application in waste management: actually, small artificial cellulosomes (minicellulosomes), for the efficient degradation of specific substrates, have been assembled *in vitro* (Bayer *et al.*, 2007).
The present study aims to express minicellulosomes containing the minimum number of rationally chosen components of the *C. cellulovorans* cellulosome, in *Lactococcus lactis*.
RESULTS: The gene encoding a truncated form of the scaffolding protein (CbpA) has been cloned and expressed in a *E. coli- L. lactis* shuttle vector under the control of the lactococcal p32 constitutive promoter. Mini-CbpA was actually secreted by the recombinant *L. lactis* strains, as demonstrated by cellulose binding assays. Cloning and expression of the genetic determinants for the cellulosomal exoglucanase S (ExgS) and endoglucanase B (EngB) of *C. cellulovorans*, as well as the construction of an artificial operon encoding a minicellulosome (miniCbpA-ExgS-EngB) is currently underway. Metabolic features of *L. lactis* cells expressing such designer cellulosomes will be tested.

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