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## INHIBITION OF P-GP/ATP<sub>ASE</sub> ACTIVITY OF RAT SMALL INTESTINE MEMBRANE VESICLES BY NOVEL DIHYDROPYRIDINE DERIVATIVES AND NOVEL N,N-DICYCLOHEXANE-4-OL-AMINE ARYL ESTERS

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Permeability glycoprotein (P-gp) is a multidrug transporter responsible for resistance to anticancer chemotherapy (MDR) and physiologically involved in absorption, distribution, and excretion of a large number of lipophilic uncharged and cationic drugs. P-gp exhibits both an ATPase activity correlated with drug transport and a basal ATPase activity in the absence of any drug. On the assumption of the existence of multiple binding sites on P-gp, both basal ATPase activity of P-gp and ATPase activity stimulated by the substrate verapamil must be taken into account when investigating P-gp modulators. P-gp recognizes numerous different classes of compounds without strict structural features including Ca<sup>2+</sup> channel modulators like 1,4-dihydropyridines, verapamil and compounds functionally related to it. To test the effects of these drugs on P-gp ATPase activity, plasma membrane vesicles were prepared from homogenates of rat small intestine mucosa (1). On these membrane vesicles containing P-gp, ATPase activity measurements were performed at 37°C by a spectrophotometric method based on continuous monitoring of ADP formation, regenerated in ATP by a coupled enzyme system consisting of pyruvate kinase/phosphoenolpyruvate and lactate dehydrogenase/NADH, according to the method of Scharschmidt et al. (2) by monitoring NADH absorbance decay with time at 340 nm. Membrane ATPases not related to P-gp, i.e. mitochondrial ATPases, Na<sup>+</sup>/K<sup>+</sup>-ATPases and Ca<sup>2+</sup>-ATPases, were inhibited by addition of 10 mM sodium azide, 0.5 mM ouabain, and 1 mM EGTA, respectively. Residual ATPase activity was fully ascribable to P-gp owing to its complete inhibition by low μM concentrations of Na-orthovanadate.

Verapamil gave a typical bell shaped concentration-activation curve with 30 μM maximum effective concentration. 3,5-dibenzoyl-4-(3-phenoxy-phenyl)-1,4-dihydro-2,6-dimethylpyridine (DP7) has been shown to inhibit at submicromolar concentrations P-gp mediated efflux of rhodamine 123 in LY5178 MDR1-transfected mouse lymphoma cells (3). It was tested at various concentrations (0.025-10 μM) either on basal or 30 μM verapamil-stimulated ATPase activities. Neither pyruvate kinase nor lactate dehydrogenase was inhibited by DP7 that, however, inhibited concentration-dependently both P-gp ATPase activities, with IC<sub>50</sub> values of about 1 μM. The inhibition mechanism of DP7 was competitive in nature, with a K<sub>i</sub> of 5.3 μM.

A number of novel isomeric N,N-dicyclohexane-4-ol-amine aryl esters, indirectly proven to be powerful inhibitors of P-gp in erythroleukemia K562 cells (4), were tested on P-gp ATPase activity. The cis-cis isomer (MC185oxa) gave a typical bell shaped concentration-activation curve with 50 nM maximum effective concentration, thus behaving like a P-gp ATPase substrate. MC185oxa was tested also on 30 μM verapamil-stimulated P-gp ATPase activity showing a bimodal effect, at concentrations ≤25 nM stimulating while at higher concentrations inhibiting it. MC176oxa, the trans-cis isomer, inhibited the basal P-gp ATPase activity in a concentration-dependent fashion up to 5 nM, at which inhibition averaged 55%; at concentrations ≥5 nM it fully antagonised the portion of ATPase activity stimulated by 30 μM verapamil, thus indicating a clear antagonism towards verapamil activation of P-gp ATPase activity. This effect needs further clarification as to its mechanism.

### References:

1. Shirazi SP, Beechey RB, Butterworth PJ (1981) *Biochem J* 194: 803-9.
2. Scharschmidt BF, Keeffe EB, Blankenship NM, Ockner RK (1979) *J Lab Clin Med* 93: 790-9.
3. Saponara S, et al. (2004) *Br J Pharmacol* 141: 415-22.
4. Teodori E, et al. (2007) *J Med Chem* 50: 599-602.

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**METABOLIC AND FUNCTIONAL EFFECTS OF ANGIOTENSIN II TYPE I RECEPTOR BLOCKERS IN EXPERIMENTAL MODELS OF DIABETES****Alfarano Chiara***Dipartimento di Farmacologia Preclinica e Clinica, Università degli Studi di Firenze, Viale Pieraccini 6, 50134 Firenze**E-mail: chiara.alfarano@unifi.it*

Losartan represents the gold standard treatment for reducing hypertension and the incidence of brain and cardiac ischemic events of diabetic patients (1-2). Since these effects occur in the absence of any modification of glucose homeostasis, one hypothesis is that losartan prevents the diabetic vascular hyper-reactivity and tissue tolerance to ischemia. We explored the effect of a losartan treatment of normo- and hyper-glycemic rats on: a) aorta hyper-reactivity to angiotensin II (AngII); b) tolerance to metabolic intoxication of isolated cardiomyocytes (3).

Cardiomyocytes and aortas were isolated from control (C), STZ-injected (55 mg/kg, D) rats not treated and treated with losartan (20 mg/kg/day in drinking water, C<sub>Los</sub> and D<sub>Los</sub>) for 3 weeks (4). Dose-response curves of AngII were performed in phenylephrine pre-contracted aortic strips (5) in the absence or in the presence of: 1) AngII receptor antagonists (irbesartan and PD123319, 1  $\mu$ M); 2) HA-1077 10  $\mu$ M, an inhibitor of ROCK1 activity. ROCK1 activity and expression were measured by indirect ELISA immunoassay and Western blot respectively. Time to rigor of cardiomyocytes superfused with a modified Tyrode's solution and field stimulated with bipolar square current pulses was followed as a surrogate of tolerance to ischemia (3). Ischemia was instituted by a solution containing NaCN (2 mmol/l) and no glucose.

Ang-II maximum effect reached 360% in group D, 250% in group D<sub>Los</sub>, and only 175% of maximum phenylephrine contracture in group C and C<sub>Los</sub>. Aortas from all groups responded similarly to irbesartan but not to HA-1077 (32% and 22% of reduction in groups D and D<sub>Los</sub>, respectively). ROCK1 enzyme activity (mU/mg of tissue proteins) was 0.04 $\pm$ 0.01 in groups C and C<sub>Los</sub>, 1.42 $\pm$ 0.22\* in group D (\*P<0.001 vs. groups C and C<sub>Los</sub>) and only 0.72 $\pm$ 0.05<sup>§</sup> (§P<0.05 vs. groups C and C<sub>Los</sub>) in group D<sub>Los</sub>. Rigor occurred significantly later in cardiomyocytes from D rats (20.50 $\pm$ 1.96 min; n=16) than in C (10.00 $\pm$ 2.03 min; n=5, P<0.001) and cells isolated from D<sub>Los</sub> rats showed similar time to rigor (9.93 $\pm$ 1.30 min; n=16) to that measured in cells from C.

In conclusion, losartan treatment reduces the hyper-reactivity to AngII in the aorta from 2-weeks diabetic rats by controlling ROCK-1 over-expression and it makes diabetic cardiomyocytes similar to normoglycemic ones in respect to their tolerance to ischemia.

## References:

1. Fournier A, Oprisiu R, Andrejak M, Fernandez N, Achard JM (2003) JAMA 289: 700.
2. Kjeldsen SE, et al. (2002) JAMA 288: 1491-8.
3. de Groot JR, et al. (2003) Cardiovasc Res 59: 705-14.
4. Raimondi L, et al. (2004) Diabetes 53: 1927-33.
5. Mazzetti L, et al. (2004) Br J Pharmacol 138: 707-15.

**VALIDATION AND PERSPECTIVES OF A NEW METHOD TO ANALYSE FREE IGF-I LEVELS IN SALIVA, AN ALTERNATIVE BIOLOGICAL MATRIX****Antonelli Giorgia**

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Insulin-like growth factors (IGFs) are peptide hormones that play a pivotal role in both embryonic and post-natal growth and metabolism. The two major IGFs are IGF-I, the more studied form, and IGF-II, less known for analytical and physiopathological application aspects. Laboratory IGF-I analysis firstly suggested only a GH-dependent effect and on well defined target districts; currently, the specific IGF-I receptor has been verified to be ubiquitous, demonstrating an autocrine and/or paracrine IGF-I role, besides having an endocrine action. IGF-I in the blood circulates free (about 5%) and bound (about 95%) to six specific binding proteins, namely IGFBP1 to IGFBP6. Plasma IGF-I measurement is mainly used as an integrated marker of GH status (1). Saliva could be an alternative matrix useful to value GH/IGF axis status, utilizing a simple non-invasive sampling procedure. Limited studies on IGF-I levels in saliva exist in the literature and RIA was the analysis method used to test IGF-I (2-4). The limited literature data are contrasting regarding the possible correlation between salivary IGF-I and serum GH. The purpose of the present study was to validate a new immunoenzymatic method to analyse salivary free IGF-I (sIGF-I).

The analysis was carried out by modifying a commercial kit for plasma matrix to measure the free IGF-I fraction. The assay method was an enzyme-immunoassay using two specific antibodies for two different IGF-I epitopes and the performance was evaluated: detection limit, precision, accuracy and specificity were defined. The biological variation and the pre-analytical variability were also investigated. Saliva samples were collected in the morning from a group of healthy volunteers to establish a reference range.

The free IGF-I calibration curve ranged from 0.05 to 5.00 µg/l. The detection limit was 0.07 µg/l. The within and between runs imprecision CVs were 10% and 13%, respectively. The recovery test, carried out with saliva spiked specimens, was 88%. The IGF-II and insulin cross reactivity was 0.1% and not detectable, respectively. The day-to-day variability demonstrated a CV of 11% in 5 consecutive days. Saliva samples can remain at either 4°C or 25°C for up to 8 h before storing at -80°C. The average level of sIGF-I was 0.18±0.06 µg/l in 20 healthy volunteers (27±3 years, 63±11 kg, 171±8 cm, 21±2 kg/m<sup>2</sup>).

The characteristics of the present method, namely sensitivity, precision, accuracy and specificity, taking into account the analytical performance parameters measured in the experimental validation tests, demonstrated its suitability for free IGF-I level measurement in human saliva specimen. The perspectives offered by this analysis stimulate further research in therapy monitoring, for example in GH-deficient children before, during and after r-hGH therapy and in acromegalic patients before, during and after therapy as with GH receptor antagonists. A toxicological purpose could also be the detection of athlete illegal use.

## Reference.

1. Le Roith D et al (2001). *Endocr Rev*, 22(1): 53-74;
2. Ryan J et al (1992). *J Clin Endocrinol Metab*, 74(4): 774-778;
3. Costigan DC et al (1988). *J Clin Endocrinol Metab*, 66(5): 1014-1018;
4. Halimi S et al (1994). *Eur J Clin Chem Clin Biochem*, 32(9): 705-707.

## DEVELOPMENT OF A NOVEL TAT- $\alpha$ -SYNUCLEIN-A30P RAT MODEL OF PARKINSON'S DISEASE FOR ASSESSING NEUROPROTECTIVE STRATEGIES

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Pathologic hallmarks of Parkinson's disease (PD) are progressive loss of dopamine neurons (DN) in the *substantia nigra pars compacta*, decreased levels of striatal dopamine (DA), and presence of cytoplasmic inclusions (Lewy bodies, LBs) in the surviving nigral DN, containing mainly aggregated  $\alpha$ -synuclein ( $\alpha$ -syn) and parkin. Motor symptoms become manifest when the striatal DN loss exceeds 70%. PD is always sporadic, except rare cases associated with genetic mutations responsible for early-onset PD: 2 missense mutations (A53T, A30P) in the  $\alpha$ -syn gene, causing dominant PD; mutations in the parkin gene, accounting for most of the familial recessive and sporadic early-onset PD cases, lacking LBs.  $\alpha$ -Syn has self-aggregation properties, increased in the mutants and upon oxidative modification. Abnormal  $\alpha$ -syn accumulation is the key pathological event in sporadic and familial PD (1). Parkin is an E3 ubiquitin-protein ligase, involved in misfolded/damaged protein degradation by proteasomes. The loss of this function is the pathogenic mechanism of early-onset PD, resulting in accumulation of parkin target proteins and deregulation of DA handling. Interactions between  $\alpha$ -syn and parkin are known: glycosylated forms of  $\alpha$ -syn, accumulating in the early-onset PD brain, and synphilin, an  $\alpha$ -syn-interacting protein present in LBs, can be ubiquitinated by parkin. In that context, it is relevant that recent *in vitro* and  $\alpha$ -syn transgenic fly studies have attributed a protective role to parkin in DN survival, thus opening attractive prospects to explore parkin as a new target for innovative PD treatment strategies, based on endogenous neuroprotection. Current neurotoxin- or gene-based mammalian animal models of PD are not appropriate to test neuroprotective strategies. In particular, neurotoxic models are limited by the fact of causing precipitous DN loss, thus reproducing only the final conditions of human PD. As the target of neuroprotective interventions is mainly early PD, when there are still cells to be protected, our research project intends to develop a novel  $\alpha$ -syn-based rat model of "mild" PD that could better approximate the early stages and progressive neurodegeneration of PD, so to be used for the evaluation of parkin neuroprotective effect. The model relies on the stereotaxic unilateral intranigral injection of the A30P mutated form of  $\alpha$ -syn fused to a protein transduction domain derived from the human immunodeficiency TAT protein (TAT- $\alpha$ -synA30P) (2), thereby allowing diffusion across the membrane and causing a localized DN loss.

The doctorate project will be performed measuring multiple outcomes in sham and treated rats: assay of TAT- $\alpha$ -synA30P and TAT-parkin brain distribution by immunohistochemistry and Western blotting; assay of DN degeneration by measuring the reduction of nigral tyrosine hydroxylase cells by immunohistochemistry and the level of striatal DA and its catabolite DOPAC by HPLC; immunohistochemistry for DA neuronal and vesicular transporter to further verify the nigrostriatal functionality; behavioural tests to evaluate impaired performance in specific motor tasks (drug-induced rotational behaviour testing for the evaluation of rotational asymmetry in animals with unilateral lesions to the DA system, using amphetamine, acting on presynaptic DA release, and apomorphine, acting as agonist at postsynaptic DA receptors; Rotarod test to evaluate motor coordination; footprint test to analyse walking patterns). Neurochemical and behavioural assays will be performed at different times (up to 1 year) following intranigral lesion. The putative neuroprotective effect of parkin on  $\alpha$ -synA30P-induced nigrostriatal neurodegeneration will be evaluated by intranigral injection of mixtures of TAT- $\alpha$ -synA30P and TAT-parkin, followed by neurochemical and behavioural assays.

### References:

1. Recchia A, et al. (2004) FASEB J 18: 617-26.
2. Albani D, et al. (2004) FASEB J 18: 1713-5.

**PPAR- $\alpha$  ACTIVATION AND GSK-3 $\beta$  INHIBITION CONTRIBUTE TO REDUCE TRANSIENT CEREBRAL ISCHEMIA/REPERFUSION INJURY IN THE RAT HIPPOCAMPUS****Benetti Elisa***Dipartimento di Anatomia, Farmacologia e Medicina Legale. Facoltà di Farmacia. Università di Torino.**E-mail: elisa.benetti@unito.it*

Cerebral ischemia/reperfusion (I/R) injury triggers a complex series of cellular and molecular mechanisms leading to tissue damage. Recent evidence suggest that the peroxisome proliferated-activated receptors (PPAR)- $\alpha$  and the glycogen synthase kinase (GSK)-3 $\beta$  are two important signalling pathways highly expressed in the central nervous system and involved in the regulation of neuronal cell survival. The activation of PPAR- $\alpha$  by both natural or synthetic ligands, such as eicosanoids and hypolipidemic fibrates, modulates target gene transcription via the formation of heterodimeric transcription factor complexes with the retinoid x receptor (1). The GSK-3 $\beta$  was first described as a component of the metabolic pathway for glycogen synthase regulation, but now it is considered to be involved in many cell functions and in the regulation of several signal transduction pathway (2). The pharmacological modulation of either PPAR- $\alpha$  or GSK-3 $\beta$  has been demonstrated to be effective in few animal models of cerebral injury (3-4). However their role in cerebral I/R injury has been investigated less extensively. Aim of this work was to study the role of these two signalling pathways in a rat model of transient cerebral I/R, focusing on the hippocampus, which is one of the brain areas most sensitive to I/R and with the highest expression level of PPAR- $\alpha$  and GSK-3 $\beta$ . Anesthetized male Wistar rats, weighing 210-230 g, were subjected to bilateral occlusion of the common carotid arteries (30 min) followed by reperfusion (1-24 h). Markers of oxidative stress were measured by spectrophotometric analysis and inflammatory markers were evaluated by Western blot analysis. Transient cerebral ischemia followed by 1 h reperfusion significantly increased the generation of reactive oxygen species, whereas the expression of inflammatory markers, such as cyclooxygenase-2 (COX-2), inducible NO synthase (iNOS) and intercellular adhesion molecule-1 (ICAM-1), was markedly increased after at least 6 h reperfusion, and lasted up to 24 h. When a highly selective PPAR- $\alpha$  agonist, WY14643 (at the dose-range 0.1-6 mg/kg i.v.), was administered 30 min prior to ischemia, reduction in oxidative stress and inflammatory response was observed, with maximal effect obtained at the dose 6 mg/kg. Similarly, pre-treatment with a highly selective GSK-3 $\beta$  inhibitor, TDZD-8 (at the dose-range 0.1-3 mg/kg i.v.), protected the brain against excessive oxidative stress and inflammatory response in a dose-dependent manner with maximal effect obtained at 1 mg/kg. Interestingly, TDZD-8 was effective also when administered during reperfusion only, pointing out both prophylactic and therapeutic effects. Moreover, TDZD-8 reduced COX-2 over-expression, while WY14643 was ineffective in the same experimental conditions. We also provided evidence that the activation of mitogen-activated protein kinases and nuclear factor- $\kappa$ B were reduced by WY14643 and TDZD-8. Overall, our data contribute to focus the possible role of PPAR- $\alpha$  activation and GSK-3 $\beta$  inhibition in cerebral I/R injury.

## References:

1. Kersten S, Desvergne B, Wahli W (2000) Nature 405: 421-4.
2. Dugo L, Collin M, Thiernemann C (2007) Shock 27: 113-23.
3. Berger J, Moller DE (2002) Annu Rev Med 53: 409-35.
4. Cohen P, Goedert M (2004) Nat Rev Drug Discov. 3: 479-87.

## ENDOTHELIUM-MEDIATED VASORELAXANT RESPONSE IN AORTIC RINGS FROM OBESE RATS

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Obesity is associated with endothelial dysfunction, an early marker of atherosclerosis (1). In clinical studies, peripheral vasomotor responses to chemical or physical stimuli are regarded as acceptable surrogate of endothelial responses (2). The evaluation of isometric tension change, before and after vasoactive molecules, of aortic rings is one of the most useful experimental methods to study endothelial function (3). To assess whether endothelial response to vasodilating agents is altered in obesity we used 2 groups of male Zucker rats, 14-15 weeks old, lean and obese. After anaesthesia, the thoracic aorta was dissected, cleaned of periadventitial fat, cut into 2.0 mm rings and mounted into baths containing warmed modified Krebs buffer for isometric tension studies. Each ring was set to normalized conditions of passive force directly determined from its circumferential length-tension relationship. Rings were contracted by phenylephrine (0.3  $\mu$ M). Vasodilatation to acetylcholine (ACh) was determined in aortic rings before and after L-NAME incubation (10  $\mu$ M), a NO synthase inhibitor. ACh dose-response curve was preserved in both lean Zucker rats (78 $\pm$ 5%, n=2), and obese (70 $\pm$ 2%, n=4). After L-NAME, ACh dose-response curve was significantly reduced in aortic rings of both obese (44 $\pm$ 25%; n=4; P<0.05) and lean (37 $\pm$ 3%; n=2; P<0.05) rats. In conclusion, in obese rats the vasorelaxing response to ACh is not different from that of lean rats.

### References:

1. Lakka TA, Lakka HM, Salonen R, Kaplan GA, Salonen JT (2001) *Atherosclerosis* 154: 497-504.
2. Kenchaiah S, et al. (2002) *N Engl J Med* 347: 305-13.
3. Angus JA, Wright CE (2000) *J Pharmacol Toxicol Methods* 44: 395-407.

## CYP2C9 AND CYP2B6 ISOFORMS AND THEIR IMPACT IN TOXICITY AND SURVIVAL IN BREAST CANCER PATIENTS

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Cytochrome p450 (CYP) is a superfamily of phase I enzymes that may be implicated in the metabolism of several antineoplastic drugs. Several subfamilies of CYP are involved in the hepatic bioactivation, through the 4-hydroxylation, of cyclophosphamide (CPA). In particular, CYP2C9 and CYP2B6 isoforms show genetic polymorphisms associated with an alteration in enzyme activity and CPA metabolism. The single nucleotide polymorphism CYP2C9\*2 (430C>T), CYP2C9\*3 (1075A>C), CYP2B6\*5(1459C>T) and CYP2B6\*7 (516G>T, 785A>G and 1459C>T) lead to a reduction of the enzyme activity.

The aim of this study was to define the role of these polymorphisms in development of severe toxicity (at the beginning and the end of therapy) and in survival in breast cancer patients after the CPA treatment.

Two hundred patients affected by breast cancer and homogeneously treated with cyclophosphamide, methotrexate, and 5-fluorouracil (CMF adjuvant regimen) have been tested on genomic DNA from peripheral blood mononuclear cells. The genotype analysis was performed using two PCR-based methods: restriction fragment polymorphism and pyrosequencing (mini-sequencing). The overall survival was studied in 185 patients.

Development of hepatic toxicity was significantly correlated with genetic variants CYP2C9\*2 and CYP2C9\*3, both after the first cycle and at the end of chemotherapy. For CYP2C9\*2 the association is confirmed when comparing patients carrying at least one 430T allele and grade 1-2-3 hepatic toxicity ( $P=0.0011$ , OR 3.55, CI 95% 1.676-7.527) with respect to the patients with 430CC genotype. A significant correlation was also found between CYP2C9\*3 and hepatic toxicity (grade 0-3) at the end of chemotherapy ( $P=0.0298$ ). This polymorphism was also significantly correlated with overall survival ( $P=0.0487$ ): patients carrying at least one variant allele show a reduced survival (Fig. 1).

Between the two isoforms studied for CYP2B6, only the variant CYP2B6\*7 had shown a significant correlation with the development of hepatic toxicity at the end of therapy ( $P=0.0039$ ). Neither CYP2B6\*5 and CYP26\*7 had a significant impact in overall survival.

The presence of genetic variants in CYP (CYP2C9\*2, CYP2C9\*3 and CYP2B6\*7) could have an impact in development of hepatic toxicity possibly due to a reduction in bioactivation of cyclophosphamide and thus in accumulation of the parental drug. Moreover, the CYP2C9\*3 polymorphism shows a significant impact in overall survival for patients with breast cancer treated with CMF chemotherapy.

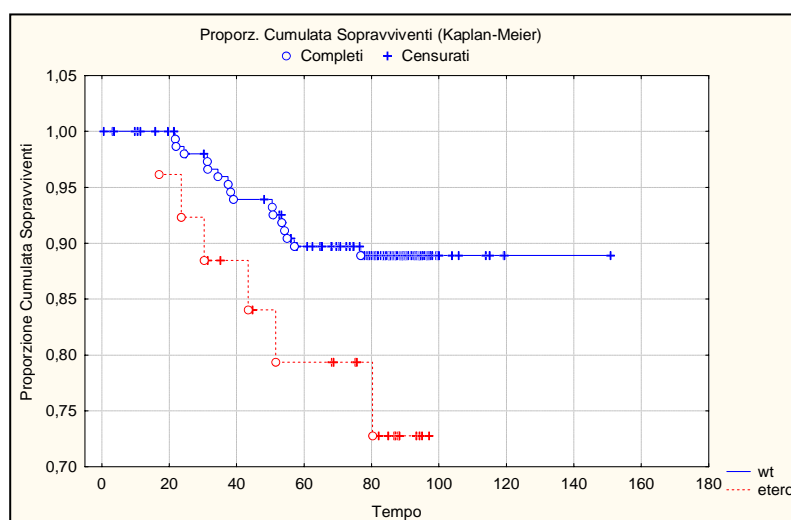


Fig. 1: Overall survival curves for CYP2C9\*3



## FUNCTIONAL DISTRIBUTION OF ECTO-ENZYMES CONTROLLING EXTRACELLULAR ADENOSINE CONCENTRATION IN THE SMALL INTESTINE OF NORMAL AND HERPES SIMPLEX VIRUS 1 (HSV1)-INFECTED RATS

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Alterations of gastrointestinal (GI) motility occur in a number of clinical conditions (e.g. inflammatory bowel disease and irritable bowel syndrome) and are characterized by smooth muscle and enteric nervous system (ENS) abnormalities. The effects exerted by adenosine (Ado) in this district depend on the expression patterns of Ado receptors (ARs) and of enzymes involved in the control of the nucleoside levels (1). In this respect, ecto-enzyme is known to play a key role. The functional expression of CD73 and ecto-adenosine deaminase (ADA) and that of ARs have been well characterized in mucosal, but not in muscular layers of the gut. The aims of the present study were to: a) verify CD73, ADA, and ARs expression in ileum strips (ISs) and intestinal longitudinal smooth muscle cells (ISMCs); b) evaluate the functional role of CD73 and ADA in Ado metabolism in these preparations; c) assess whether Herpes Simplex Virus 1 (HSV1) latent infection causes changes in CD73 and ADA distribution in rat ileum. ISMCs were prepared by primary explant from freshly isolated rat ileum (2). Protein immunofluorescence (IF) studies were performed on formaldehyde-fixed tissue sections and cells, and detected by confocal fluorescent microscopy. For evaluating enzyme activity, ISs incubated in Tyrode solution or cultured ISMCs were treated with exogenous AMP (50  $\mu$ M), the direct precursor of Ado. Incubation media were sampled at 0.5, 5, 15, 30, and 60 min and AMP, Ado, and inosine (Ino) were detected by HPLC. A model of chronic HSV1 infection that alters GI motility in rats (3) has been used. In full-thickness sections, IF revealed the constitutive presence of ADA and all 4 AR subtype in the rat ileum with more intensive staining on the mucosal layer. Mucosa and *muscularis externa* exhibited immunopositivity for CD73, which showed a regional distinct distribution. In ISMCs, IF localized ADA and ARs mainly in the cytosolic compartment, while showing CD73 staining only on the cell surface, where the ecto-enzyme partially co-localized with ZO-1 and vinculin at the level of intercellular junctions. AMP added to the incubation medium of ISs was metabolized by 90% within 5 min. The nucleotide was completely cleared from the medium after a 30 min incubation ( $t_{1/2}$ =0.3 min). Preincubation of ISs with 200  $\mu$ M  $\alpha,\beta$ -methylene ADP (AOPCP), a CD73 inhibitor, allowed the recovery of 43% and 10% of exogenously added AMP at 5 and 60 min of incubation, respectively. AOPCP also prevented the concomitant increase in Ado and Ino. In ISMCs, AMP clearance was less rapid ( $t_{1/2}$ =4.1 min) and the nucleotide was still detectable after 60 min ( $2.8\pm 0.2$   $\mu$ M). These changes were associated with a marked increase in Ado without significant modifications in Ino levels, suggesting low ADA activity on the cell surface. Increasing AMP concentration up to 100  $\mu$ M did not alter CD73 kinetics. Treatment of ISMCs with AOPCP (200  $\mu$ M) or concanavalin A (0.1 mg/ml), a non-nucleoside lectin CD73 inhibitor, reduced the elimination of exogenous AMP to a similar extent ( $t_{1/2}$ =9.9 and 9.7 min, respectively), in parallel with Ado formation. Moreover, the presence of levamisole (10 mM), an alkaline phosphatase inhibitor, did not modify AMP levels in the incubation medium, providing evidence that ISMCs are capable of converting AMP to Ado solely by means of CD73. HSV1 latency in rat ENS was associated with modified expression of CD73 and ADA during infection. CD73 appears to be the major enzyme responsible for generation of extracellular Ado from exogenous AMP. In ISMCs its surface localization may reflect an important way for cell-cell communications and cell-environment interactions. Distribution and functional activity of CD73 and ADA represent a crucial point for modulating Ado levels in the gut and for the fine activation of low and high affinity ARs controlling GI motility and neurotransmission. The altered expression of these enzymes in HSV1-infected intestine suggests their involvement in inflammatory and immune responses. The Ado system may thus represent a target for pharmacological intervention in GI disorders.

### References:

1. Bours MJ, Swennen EL, Di Virgilio F, Cronstein BN, Dagnelie PC (2006) *Pharmacol Ther* 112: 358-404.
2. Khan I, Blennerhassett MG, Kataeva GV, Collins SM (1995) *Gastroenterology* 108: 1720-8.
3. Oppellaro C, et al. (2007) 33° Congresso Nazionale SIF, Abstracts Book 359.

## DRUG METABOLISM MODULATION BY “INTEGRATED” FRUIT AND CHEMOPREVENTION

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A great deal of epidemiological, animal and *in vitro* studies suggest an inverse association between fruit and vegetable intake and cancer. Healthy benefit seems to be ascribed to phytochemical, non-nutrient present in the green matrix. Among these, polyphenols, thanks to their (direct and) indirect antioxidant properties, are considered one of the most promising anticancer agents. Up-regulation of phase-II together with down-regulation of phase-I xenobiotic metabolizing enzymes (XMEs) is in fact one of the most evoked way of cancer control. Since peaches are particularly rich in polyphenols, here the XME-based anticancer dietary manipulation by supplementing male Sprague Dawley rat diet with *in toto* peach lyophilized was investigated.

Six animals for each treatment group were used. Rats received by oral gavage 250 or 500 mg/kg b.w. of peach lyophilized twice a day for 7 or 14 consecutive days. Liver subcellular preparation were tested for various cytochrome P450 (CYP) linked-monooxygenases either by using specific substrates as probes of different isoforms or testosterone as multibiomarker. Phase-II supported XMEs such as glutathione S-transferase (GST) and UDP-glucuronosyl transferase (UDPGT) were also studied.

Peach treatment generated a complex pattern of induction (up to 1.8-fold for UDPGT and 1.6-fold for CYP2B1, after 14 and 7 day, respectively;  $P < 0.01$ ) and suppression (up to 64% for GST and 30% loss for NADPH (P450) c reductase activities, after 14 and 7 day, respectively;  $P < 0.01$ ) of various phase-I and -II XME linked activities. The use of testosterone as multibiomarker confirmed the generalized suppressive effect of peach lyophilized on phase-I XMEs.

The modified metabolic fingerprint could, in part, explain the protective effects of the peach: the decreased bioactivation/detoxification ratio would protect from neoplasia enhancing “carcinogen clearance”. Nevertheless, the recorded manipulation of microsomal metabolism suggest, if extrapolated to humans, that caution should be exercised before regular single green mass consumption. Because of the XME complexity, several unhealthy consequences can stem from enzyme modulation. Noteworthy, public health alimentary programs promote wise colourful fruit and vegetable intake.

**IMPAIRMENT IN VASCULAR HYDROGEN SULFIDE PRODUCTION IN DIABETIC CONDITIONS****Brancaleone Vincenzo***Dipartimento di Farmacologia Sperimentale, Facoltà di Farmacia, Università degli Studi di Napoli "Federico II", via D. Montesano 49, 80131 Napoli**E-mail: v.brancaleone@unina.it*

Hydrogen sulphide (H<sub>2</sub>S) is a gas endogenously generated from L-cysteine (L-Cys) by cystathionine-β-synthase (CBS) and cystathionine-γ-lyase (CSE). CBS and CSE are widely distributed in tissues; to date CBS is a predominant source of H<sub>2</sub>S in the central nervous system, whereas CSE is a major H<sub>2</sub>S-producing enzyme in the cardiovascular system (1-2). Several papers have shown an involvement of H<sub>2</sub>S in the cardiovascular system, however the significance of H<sub>2</sub>S in vascular homeostasis is not yet completely understood (3). Almost all the studies performed at the present stage deals with normal vessels and their response to exogenous H<sub>2</sub>S. As cardiovascular diseases are often associated with endothelial dysfunction, we have investigated whether under pathological conditions there is an alteration of H<sub>2</sub>S pathway too. In order to address this issue, we used a model of vascular dysfunction e.g. NOD mice, a strain that develops spontaneous diabetes mellitus. In these animals the hyperglycaemic state is associated to a progressive endothelial dysfunction that involves a reduced ACh-induced vasorelaxation as well as an alteration of the adrenergic system (4). Mice were divided into three groups according to the different stages of pathology (NOD-I: diabetes onset; NOD-II: diabetic; NOD-III: severe diabetes). In order to study the vascular response to H<sub>2</sub>S, we have used as stimulus NaHS, a well characterised source of H<sub>2</sub>S, while L-Cys was used to evaluate the residual ability of the tissue to efficaciously convert L-Cys to H<sub>2</sub>S. In control and NOD I mice, that have normal glycosuria, the relaxant response induced by NaHS on isolated aorta was similar. The disease progression leads to an increase in NaHS-induced vasodilatation in NOD II that becomes statistically significant in NOD III. These data suggest that when the disease is well established, the damaged vessels, where a residual activity of the endothelium is still present, increase smooth muscle cells (SMCs) ability to respond to exogenous H<sub>2</sub>S, suggesting that SMCs represent the target of H<sub>2</sub>S. We found an opposite profile when we used L-Cys. L-Cys-induced vasorelaxation significantly declined following the pathology progression. This event that parallels the loss of activity of ACh-induced vasorelaxation further suggest that the endothelium plays a role in the biochemical pathway leading to the conversion of L-Cys to H<sub>2</sub>S. To further gain inside in this mechanism and to test the effect of high glucose concentration on L-Cys/H<sub>2</sub>S pathway, we used bovine aortic endothelial cells (BAEC) in a normal (NG) and high glucose (HG) environment (5). BAEC incubated in NG produce low levels of H<sub>2</sub>S, while a significant increase in H<sub>2</sub>S levels was found when stimulated with L-Cys. Conversely, although high glucose does not affect significantly basal H<sub>2</sub>S production, the hyperglycaemic condition abrogates the ability of BAEC to metabolise L-Cys to H<sub>2</sub>S. In conclusion our data suggest that pathological conditions such as vascular disorders do not affect the ability of vascular tissue to respond to H<sub>2</sub>S, while dramatically reduce its ability to produce H<sub>2</sub>S.

## References:

1. Wang R (2002) FASEB J 16:1792-8.
2. Lowicka E, Beltowski J (2007) Pharmacol Rep 59: 4-24.
3. Bhatia M (2005) IUBMB Life 57: 603-6.
4. Bucci M, et al. (2004) Arterioscler Thromb Vasc Biol 24: 721-6.
5. Steiner G (1981) Diabetes 30: 1-7.

## METABOLISM OF ADENINE NUCLEOTIDES BY EXO-ENZYMES RELEASED IN THE INCUBATION MEDIUM OF RAT ILEUM

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Extracellular nucleotides play a relevant role in inflammation and other pathologic conditions. Also their breakdown product, adenosine (Ado), is an important modulator of physiological responses during ischaemia, ipoxia, and inflammatory processes (1). Considering the role of ATP and Ado in the pathophysiology of gastro-intestinal tract (2), we investigated the possible release of enzymes involved in purine metabolism [NTPDase, phosphodiesterase, 5'-nucleotidase, and Ado deaminase (ADA)] during *in vitro* incubation of rat ileum.

Longitudinally cut strips (3 cm) from distal ileum of male Wistar rats were incubated in vials containing 2 ml of aerated (95% O<sub>2</sub>, 5% CO<sub>2</sub>) and warmed (36.5°C) Tyrode solution. Conditioned medium was obtained by removing the untreated tissue after 30min incubation. ATP 50 μM, cAMP 50 μM, AMP 50 μM or Ado 50 μM were added to conditioned medium in the absence or in the presence of enzyme inhibitors. An HPLC method [modified from (3)] was used to quantify purine nucleotides and their metabolites in the samples. Statistical analysis was performed by one-way ANOVA, followed by Newman-Keuls multiple comparison test.

Exogenous Ado returned to basal levels (0.20±0.04 μM) within 5 min of incubation in conditioned medium, and the sum of its metabolites (inosine and hypoxanthine) roughly matched the initial amount of the added nucleoside. The ADA inhibitor, erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA; 1 μM), added 15 min before Ado, allowed full recovery of the nucleoside in the next 2 min of incubation, indicating that its elimination is brought about by *exo*-ADA. ATP and AMP were completely cleared from the conditioned medium of rat ileum within 60 min, and their changes were quantitatively correlated with the increase of their metabolites: Ado, inosine, and hypoxanthine. Thus also NTPDase and 5'-nucleotidase are released from ileum tissue. By contrast, the concentration of exogenous cAMP did not change in 60 min, suggesting that cAMP-phosphodiesterase is not released by rat ileum as *exo*-enzyme.

Adenine nucleotides have powerful immunoregulatory effects (4) and ATP may also be beneficial in the treatment of intestinal disorders in which permeability changes are involved (5); in the intestine, reparative effects of exogenous nucleotides have been reported in different pathologies, such as diarrhoea, enterocolitis and bowel resection (6). Agents that increase purine concentrations in the local environment such as hydrophilic, membrane impermeable inhibitors of purine metabolizing enzymes could be successful in treating inflammatory, autoimmune and acute ischemic bowel diseases.

### References:

1. Bours MJ, Swennen EL, Di Virgilio F, Cronstein BN, Dagnelie PC (2006) *Pharmacol Ther* 112: 358-404.
2. Kuno M, et al. (2006) *Eur J Pharmacol* 534: 241-9.
3. Childs KF, Ning XH, Bolling SF (1996) *J Chromatogr B Biomed Appl* 678: 181-6.
4. Haskó G, Cronstein BN (2004) *Trends Immunol* 25: 33-9.
5. Bours MJ, Troost FJ, Brummer RJ, Bast A, Dagnelie PC (2007) *Eur J Gastroenterol Hepatol* 19: 245-50.
6. Arnaud A, López-Pedrosa JM, Torres MI, Gil A (2003) *J Pediatr Gastroenterol Nutr* 37: 124-31.

## ROLE OF CREB IN NOP GENE EXPRESSION REGULATION BY $\Delta^9$ -THC IN SH-SY5Y CELLS

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CREB (cAMP response element-binding) protein is a transcription factor, which binds to specific DNA sequences named cAMP response elements (CRE) in the regulatory regions of specific target genes (1). CREB represents a site of convergence where different signalling pathways and their associated stimuli produce plasticity by altering gene expression (2). Regulation of gene expression is one mechanism by which opioids, cannabinoids and other drugs of abuse can induce relatively long-lasting changes in the brain (neuronal plasticity). The neuropeptide nociceptin/orphanin FQ (N/OFQ) is the endogenous ligand for NOP receptor and acts as a functional anti-opioid peptide (3). Opioids and cannabinoids are psychoactive drugs sharing a common pharmacological profile (4-6). We previously reported that the exposure to  $\Delta^9$ -THC produced a dose-dependent down-regulation of NOP receptor binding and gene expression in SH-SY5Y cells.

On this basis, the aim of our study was: first, to verify that the NOP gene expression-down regulation may be mediated by CB1 receptor activation; second, to investigate the effects of  $\Delta^9$ -THC on phospho-Ser133 CREB levels in SH-SY5Y cell line, to correlate this transcription factor with the alterations of NOP mRNA levels observed.

The cells were exposed to 50-100-150-200 nM  $\Delta^9$ -THC for 24 h. The CB1 receptor antagonists SR141716A (1  $\mu$ M) e AM251 (1  $\mu$ M) were added to the cell culture medium 20 min before adding  $\Delta^9$ -THC for 24 h. To investigate gene expression, cultured cells were collected, lysed in 1 ml of OMNIZOL and total RNA was extracted according to the manufacturer protocols and dissolved in DNase and RNase free water. RT-PCR was carried out according the SuperScript First strand Synthesis System for RT-PCR (Invitrogen). To evaluate phospho-Ser133 CREB levels cultured cells were collected, sonicated in lysis buffer (50 mM Tris pH 7.5, 500 mM NaCl, 2 mM  $\text{Na}_3\text{VO}_4$ , 10  $\mu$ M  $\text{Na}_4\text{P}_2\text{O}_7$ , 10 mM EDTA, 0.25 mM PMFS, 2 mM EGTA, 1% TRITON, 0.02%  $\text{NaN}_3$ , 20  $\mu$ g/ml leupeptin, 10  $\mu$ g/ml aprotinin, 10  $\mu$ g/ml pepstatin) and the protein concentration was determined by the Bradford protein assay. Western blot analysis was carried out according the manufacturer protocols (Cell Signalling). RT-PCR analysis showed that the decrease of NOP receptor mRNA levels after  $\Delta^9$ -THC exposure was completely blocked by pretreatment with CB1 receptor antagonists SR141716A (100.0 $\pm$ 1.8, 90.3 $\pm$ 5.3, 92.0 $\pm$ 11.5, 89.8 $\pm$ 7.9, 94.9 $\pm$ 12.5 and 112.5 $\pm$ 12.5 vs. controls 100%, mean $\pm$ SE of five independent experiment for cells exposed to 50-100-150-200 nM  $\Delta^9$ -THC+SR141716A 1  $\mu$ M and to SR141716A 1  $\mu$ M alone) and AM251 (100.0 $\pm$ 1.2, 91.7 $\pm$ 3.1, 100.6 $\pm$ 10.1, 110.5 $\pm$ 3.3, 101.1 $\pm$ 3.2 and 116.8 $\pm$ 14.3 vs. controls 100%; mean $\pm$ SE of five independent experiment for cells exposed to 50-100-150-200 nM  $\Delta^9$ -THC+AM251 1  $\mu$ M and to AM251 1  $\mu$ M alone). Western blot analysis showed a significant decrease in CREB phosphorylation at Ser133 in cells exposed to 50-200 nM  $\Delta^9$ -THC (100.0 $\pm$ 4.0, 99.2 $\pm$ 3.3, 90.7 $\pm$ 5.7, 79.6 $\pm$ 5.5\* and 81.7 $\pm$ 5.4\* vs. controls 100%, \*P<0.05, mean $\pm$ SE of five independent experiment). Moreover no changes were observed in total CREB (100.0 $\pm$ 2.3, 99.1 $\pm$ 4.1, 99.7 $\pm$ 4.3, 105.7 $\pm$ 6.9 and 108.0 $\pm$ 5.5 vs. controls 100%, mean $\pm$ SE of five independent experiment).

These results show that the NOP gene expression down-regulation induced by  $\Delta^9$ -THC is mediated by CB1 receptor activation. In addition our data demonstrate that  $\Delta^9$ -THC is able to induce a decrease of CREB phosphorylation. Taken together, these data suggest that the decrease of phospho-Ser133 CREB levels could elicit the inhibition of the transcriptional cascade of NOP gene expression, which in turn could induce the NOP gene expression down-regulation in SH-SY5Y cells.

### References:

1. Nestler EJ (2001) Nat Rev Neurosci 2: 119-28.
2. Shaywitz AJ, Greenberg ME (1999) Annu Rev Biochem 68: 821-61.
3. Meunier JC (1997) Eur J Pharmacol 340: 1-15.
4. Tanda G, Pontieri FE, Di Chiara G (1997) Science 276: 48-50.
5. Braida D, Pozzi M, Parolaro D, Sala M (2001) Eur J Pharmacol 413: 227-44.
6. Cichewicz DL (2004) Life Sci 74: 1317-24.

## ALDEHYDE DEHYDROGENASE 7A1 (ALDH7A1) PROTECTS AGAINST OSMOTIC STRESS-INDUCED APOPTOSIS

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Aldehyde dehydrogenase 7A1 (ALDH7A1) is a NAD<sup>+</sup>-dependent enzymes that shows a high degree of evolutionary conservation in plants and animals. In plants, ALDH7A1 protects cells from toxic insults and is highly expressed during osmotic stress, suggesting that this protein may be involved in turgor pressure regulation. Despite this, the role of ALDH7A1 in mammalian cells is still controversial. Human ALDH7A1 is expressed in a wide variety of tissues, specifically in the cochlea of the ear, in the kidney, and in the cardiovascular system. Previous studies have demonstrated that ALDH7A1 may influence cell turgor pressure in whole cochlea, but its functional significance in protecting cells against osmotic stress has not yet completely elucidated. As widely documented in literature, in mammalian renal cells the response to hyper tonicity induced by NaCl involves adaptive accumulation of several osmolytes as well as increased expression of genes and proteins that confer protection to cells. The purpose of the present study was to investigate the role of ALDH7A1 in NaCl-induced osmotic stress and apoptosis.

The toxicity of NaCl was evaluated in chinese hamster ovarian (CHO) cells transfected with ALDH7A1 and compared with CHO cells mock transfected with vector only, using MTT and Alamar blue assays, while the apoptosis was analyzed with DNA fragmentation. In order to evaluate the oxidized proteins concentration as index of oxidative stress, we measured protein carbonyls levels.

Both CHO-Vector and CHO-ALDH7A1 cells demonstrate cell shrinkage, reduced cell density and cell death. However, CHO-ALDH7A1 cells were affected less by NaCl treatment, proving lower cell damage and increased cell viability when compared to CHO-Vector cells. DNA fragmentation, a characteristic of apoptosis, was observed in CHO-Vector cells after NaCl treatment at 200 and 400 mM. In contrast, CHO-ALDH7A1 cells did not demonstrate DNA laddering at either concentration of NaCl. Likewise, NaCl-induced decrease in cell viability, as measured by the MTT assay, was more evident in CHO-Vector than in CHO-ALDH7A1 cells. Nevertheless, the protein carbonyls assay did not show any significant difference between CHO-ALDH7A1 and CHO-Vector cells.

In conclusion, ALDH7A1 appears to confer protection to CHO cells from NaCl-induced cytotoxicity and apoptosis, suggesting a possible role for ALDH7A1 in a cell defence mechanism against NaCl-induced osmotic stress and apoptosis. We are now extending the research field also to cardiovascular cells, in order to figure out the specific role of ALDH7A1 protein in mammalian system and to investigate a some involvement in oxidative stress.

## THE EFFECTS OF DOXORUBICIN ON RAT CARDIAC STEM CELLS (CSCS)

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Doxorubicin (DOX), an anthracycline and antibiotic drug, is one of the most active antineoplastic agents, but its clinical application is limited by the risk of developing dilated cardiomyopathy and congestive heart failure. The pathophysiological mechanisms leading to the development of anthracycline-induced cardiomyopathy are not completely understood. It has been shown that DOX induces apoptosis by activation of reactive oxygen species (ROS) as a consequence of the formation of an iron-DOX complex. ROS are able to modulate the activity of several kinases (p38 MAPK) and transcription factors (NF- $\kappa$ B) that regulate the cell cycle and pro/anti-apoptotic pathways (1). The paradigm of the heart as a post-mitotic organ has been challenged by the recognition of cardiac stem cells (CSCs) that express surface antigens c-kit, MDR1, sca-1. CSCs proliferate and differentiate into cardiac lineages, control myocardial cell turnover, and are implicated in tissue homeostasis and cardiac repair (2).

The hypothesis has been advanced that the cardiotoxicity of DOX can be related to the negative impact of the drug on the CSCs population. To determine whether the treatment with DOX can affect cardiac primitive cells, rat clonogenic CSCs were cultured and exposed to different concentrations of DOX (0.1, 0.5, and 1  $\mu$ M) for a period of 12, 24 and 48 h. Cell viability was evaluated by MTT assay and CSCs proliferation was assessed by BrdU incorporation and the expression of the marker of mitosis, phospho-histone H3. The oxidative damage of DNA was measured by the detection of the product of ROS, 8-hydroxy-deoxy-guanosine (8-OHdG), and apoptotic cell death was quantified by TUNEL assay.

The treatment with DOX affected cell viability and proliferation in a time- and dose-dependent manner. MTT test showed a significant decrease in CSCs vitality at 24 h [0.5  $\mu$ M=76%, 1  $\mu$ M=67%;  $P<0.05$  vs. control (CTL)] and at 48 h (0.5  $\mu$ M=34%, 1  $\mu$ M=10%;  $P<0.05$  vs. CTL). Additionally, BrdU incorporation and the expression of phospho-histone H3 were markedly reduced (BrdU incorporation at 24 h: CTL=42%, 0.5  $\mu$ M=28%, 1  $\mu$ M=24%;  $P<0.05$  vs. CTL; at 48 h (CTL=43%, 0.5  $\mu$ M=18%, 1  $\mu$ M=17%;  $P<0.05$  vs. CTL. Phospho-histone H3 at 24 h: CTL=10%, 0.5  $\mu$ M=7%, 1  $\mu$ M=5%;  $P<0.05$  vs. CTL; at 48 h CTL=10%, 0.5  $\mu$ M=4%, 1  $\mu$ M=3%;  $P<0.05$  vs. CTL)). Importantly, DOX induced oxidative stress, DNA damage, and apoptosis in CSCs. The significant increase in fraction of cells positive for 8-OHdG was time- and dose-dependent (12 h: CTL=0%, 0.5  $\mu$ M=4%, 1  $\mu$ M=7%;  $P<0.05$  vs. CTL; 24 h: CTL=0%, 0.5  $\mu$ M=7%, 1  $\mu$ M=10%;  $P<0.05$  vs. CTL; 48 h: CTL=0%, 0.5  $\mu$ M=14%, 1  $\mu$ M=14%;  $P<0.05$  vs. CTL). Apoptotic cell death followed a similar pattern (12 h: CTL=0%, 0.5  $\mu$ M=2%, 1  $\mu$ M=6%;  $P<0.05$  vs. CTL; 24 h: CTL=0%, 0.5  $\mu$ M=9%, 1  $\mu$ M=20%;  $P<0.05$  vs. CTL; 48 h: CTL=0%, 0.1  $\mu$ M=2%, 0.5  $\mu$ M=9%, 1  $\mu$ M=24%;  $P<0.05$  vs. CTL).

In conclusion, the treatment with DOX produces an oxidative stress and has an impact on growth and death of CSCs. In addition to cardiomyocytes and endothelial cells (3), CPCs is the new cardiac cell population negatively affected by DOX, and DOX exposure can lead to a progressive reduction of the CSC pool. The better understanding of underlying mechanisms can lead to new therapeutic strategies for anthracycline-induced cardiomyopathy and heart failure.

### References:

1. Minotti G, Menna P, Salvatorelli E, Cairo G, Gianni L (2004) *Pharmacol Rev* 56: 185-229.
2. Beltrami AP, et al. (2003) *Cell* 114: 763-76.
3. Wang S, et al. (2002) *Biochem J* 367: 729-40.

**BRAIN XENOBIOTIC METABOLISM: CYTOCHROME P450 ACTIVITY TOWARD PROBE SUBSTRATES IN RAT BRAIN MICROSOMAL PREPARATIONS****Caradonna Nicola Pasquale***Drug Profiling Unit, Siena Biotech S.p.A., via Fiorentina 1, 53100 Siena  
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Although the overall cytochrome P450 (CYP) level in the brain is approximately 0.5-2% of that in liver microsomes (1) it can play an important role in therapeutic and side-effects response to centrally acting drugs (2). Furthermore, CYPs are present in the brain regions at different concentrations and isoform distribution; possibly contributing to incorrect prediction of brain xenobiotic metabolism when liver data is used.

The objective of the current study was to investigate the metabolism of CYP marker substrates in rat brain microsome preparations, in comparison to their liver metabolism.

The preparation of rat brain microsomes followed the procedure described by Dragoni et al. (2003) (3). Briefly, after removal of meninges and blood vessels almost five brains were pooled and homogenized in 0.1 M Tris, containing 0.1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 0.2 mM EDTA, 1.15% KCl, and 20% (v/v) glycerol at pH 7.4. The homogenate was centrifuged twice at 17,000 g for 30 min. The supernatant obtained was centrifuged at 100,000 g for 1 h. The resulting pellet was suspended in the homogenization buffer, without phenylmethylsulfonyl fluoride and stored at -80°C until use. The yield of microsomes from the brain was relatively low (1.5-2 mg of microsomal protein per g of brain). Two mg/ml of microsomal preparations were incubated with 3 concentrations of marker substrates (testosterone, dextromethorphan, diclofenac, and chlorpromazine). The parent and metabolite compounds were measured by UPLC/MS system [Waters Acquity UPLC chromatograph connected to a Waters LCT Premier time-of-flight (TOF) mass spectrometer equipped with an Electrospray (ESI) ion source]. The run-to-run duration time of analysis, performed with a rapid gradient, was 6.5 minutes.

CYP-dependent testosterone hydroxylations are regio- and stereo-specific reactions catalyzed by different CYP isozymes. Our data showed that the CYP activities present in rat brain microsomes foster a different metabolite pattern with respect to those observed in liver. The incubation of 50 µM testosterone with liver microsomes contributes to the formation of 2α-, 6β-, and 16α-hydroxytestosterone as principal metabolites (characteristic of CYP3A and 2B) at the rate of 308, 92, and 472 pmol/min/mg proteins. However, rat brain microsomes mainly produced the 6α-, 6β-, 2β-, and 16β-hydroxytestosterone (related to 2A, 2B, and 3A isozymes), with an activity more than 2 orders of magnitude lower than liver (0.56, 0.17, 0.12, and 0.34 pmol/min/mg proteins).

In a similar manner, dextromethorphan O- and N-dealkylation activities (associated with 2D and 3A family) were observed in brain microsomes, but the formation rate of metabolites, at 100 µM dextromethorphan, was about 3 order of magnitude lower than the activity observed in liver (0.47 and 0.36 pmol/min/mg proteins, and 352 and 416 pmol/min/mg proteins, respectively). When diclofenac was studied as a probe substrate, no metabolite was detected in brain microsomes in our experimental conditions.

The rates of formation of nor-chlorpromazine (0.13 pmol/min/mg proteins), hydroxyl-chlorpromazine (0.01 pmol/min/mg proteins), and chlorpromazine sulfoxide (0.42 pmol/min/mg proteins), in the presence of rat brain microsomes and at 10 µM chlorpromazine, was about 0.08%, 0.04%, and 0.5% of the metabolism rate in liver microsomes.

Our data indicate that xenobiotic CYP-dependent metabolism is still active in brain and can play a crucial role in the therapeutic and/or toxicological effects of drugs. Furthermore, incubation conditions and analytical methods employed here are suitable for high-throughput screening of the brain metabolism of new drugs.

## References:

1. Miksys SL, Tyndale RF (2002) J Psychiatry Neurosci 27: 406-15.
2. Meyer RP, Gehlhaus M, Knoth R, Volk B (2007) Curr Drug Metab 8: 297-306.
3. Dragoni S, et al. (2003) J Neurochem 86: 1174-80.



**EFFECTS OF THE GLYCOGEN SYNTHASE KINASE-3 $\beta$  INHIBITOR, TDZD-8, IN A DIABETIC RAT MODEL OF ISCHEMIA/REPERFUSION INJURY****Castiglia Sara***Dipartimento di Anatomia, Farmacologia e Medicina Legale. Facoltà di Farmacia. Università di Torino,**E-mail: 316260.dottorato@unito.it*

In 1980, Embi et al. (1) identified glycogen synthase kinase (GSK)-3 [E.C. 2.7.11.1] as one of the protein kinases able to phosphorylate and inactivate glycogen synthase. Initially, the functions of GSK-3 were thought to be limited to glycogen metabolism; however, 20 years after its discovery, this kinase is known to be involved in the regulation of many cell functions and of several signal transduction pathways (2). Two mammalian isoforms of GSK-3 are known: GSK-3 $\alpha$  and  $\beta$ . They are encoded by distinct genes, with molecular weights of 51 and 47 kDa, respectively, high homology and different tissue distribution (3). GSK-3 $\beta$  is highly expressed in the central nervous system, particularly in the hippocampus (4). It is constitutively active and it is inactivated by phosphorylation at serine 9 (Ser<sup>9</sup>) of its N-terminal. Insulin is an example of endogenous inhibitor of GSK-3 $\beta$  and highly selective synthetic inhibitors have been recently developed (5). As some evidences suggest that GSK-3 $\beta$  inhibition may protect against cerebral ischemia/reperfusion (I/R) injury (6) and being diabetes an important risk factor for cardiovascular events, among which transitory ischemic attacks (7), the present work aims to investigate the effects of the pharmacological inhibition of GSK-3 $\beta$  against cerebral I/R injury in diabetic rats. We used the selective GSK-3 $\beta$  inhibitor TDZD-8 (4-benzil-2-methyl-1,2,4-thiadiazolidine-3,5-dione) and we focused our attention on hippocampus as the brain area most sensitive to I/R injury and with the highest expression of GSK-3 $\beta$ . Male Wistar rats (n=37), were subjected to bilateral occlusion of common carotid arteries for 30 min, followed by 1 or 24 h reperfusion. Diabetes was induced with a single i.v. injection of streptozotocin (STZ) (65 mg/kg) administered 4 weeks before I/R. Rats were treated, at the beginning of reperfusion, with TDZD-8 (0.1-3 mg/kg i.v.) to evaluate its therapeutic effects. Hippocampus was isolated from the brain and homogenated for biochemical assays and spectrophotometric analysis.

As shown by RT-PCR and Western blot analysis, total GSK-3 $\beta$  expression in the hippocampus of diabetic rats was higher than that recorded in non-diabetic animals and it was not modified by I/R. TDZD-8 administration caused the Ser<sup>9</sup> phosphorylation and hence inactivation of GSK-3 $\beta$  in a dose-dependent manner, with maximal effect observed at 3 mg/kg. Diabetic animals that underwent I/R presented higher production of reactive oxygen species and expression of inflammatory markers in comparison with non-diabetic animals. All these parameters decreased following TDZD-8 administration. Although the signalling pathways involved in our observation has to be better elucidated, our preliminary data suggest that GSK-3 $\beta$  inhibition may contribute to protect against cerebral I/R injury in the presence of diabetes.

## References:

1. Frame S., Cohen P. (2001) *Biochem. J.* 359:1-16.
2. Woodgett JR (1990) *EMBO J* 9: 2431-8.
3. Woodgett JR, Ohashi PS (2005) *Nat Immunol* 6: 751-2.
4. Cohen P, Goedert M (2004) *Nat Rev Drug Discov* 3: 479-87.
5. Collino M, et al. (2007) *Br J Pharmacol* submitted
6. Kernan WN, et al. (2003) *Neurology* 60: 1447-51

## STRAIN-RELATED EFFECTS OF POST-WEANING SOCIAL ISOLATION ON BIOGENIC AMINE CONTENT IN POSTMORTEM ADULT RAT BRAIN

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Animal models are important tools in the study of human diseases and design of new treatments. In the case of mental disorders it is difficult to develop an animal paradigm that may elicit behavioural and neurochemical changes well related to human disorders. It has been widely demonstrated that the isolation rearing of rats (1), provides a non-pharmacological and developmental specific method of inducing schizophrenic-like behavioral deficits (2). It has been also reported that isolation-induced deficits in prepulse inhibition could be detected in Lister Hooded (LH) but not in Wistar (W) rats (3) and it has been supposed that this could be attributed to the different rat strain.

Our study was focused on describing changes in adult neurochemistry in both Wistar (W) and Lister Hooded (LH) rats following postweaning social separation by analysing biogenic amine content in postmortem adult brain regions such as prefrontal cortex (PFC), nucleus accumbens (NAC), striatum (St) and hippocampus (HIP). In addition, we realized a comparison of neurochemical basal conditions in these two different rat strains.

Our results have shown several significant strain-related differences in basal biogenic amine content of the brain regions considered. In particular, W rats showed decreased levels of dopamine (DA) in all the examined areas. In PFC, NAC and HIP of W rats it was evidenced increased levels of serotonin (5-HT) when compared to LH, while the striatal levels of 5-HT were decreased. Furthermore, W rats showed decreased concentrations of noradrenaline (NA) in PFC and HIP and increased levels of NA in NAC with respect of LH animals.

Moreover, we observed several effects of isolation rearing on the post-mortem tissue concentration of biogenic amines, both in W and in LH rats. In particular, in PFC of ISO W were detected lower levels of DA and an enhanced value of the 5-HT turnover when compared to GRP. On the contrary, isolation rearing increased DA and homovanillic acid (HVA) concentration in PFC of LH animals. The striatal levels of 5-HT were also decreased in ISO W rats, while the striatal turnover of 5-HT resulted enhanced by the social deprivation. In W NAC, social isolation reduced DA, HVA, 3,4-dihydroxyphenilacetic acid (DOPAC), 5-HT and NA levels, while it increased the 5-HT turnover. There were no significant neurochemical alterations in St and NAC of ISO LH rats with respect to GRP. In W rats the hippocampal levels of DA and 5-HT were significantly decreased by isolation rearing, while, in the same brain area, ISO LH animals showed an increase of DOPAC levels, DOPAC/DA and (DOPAC+HVA)/DA ratios.

In conclusion, these data provide, for the first time, evidence that W and LH have different basal neurochemical profile and that isolation rearing in the rats results in different strain-dependent brain areas dysfunction.

### References:

1. Weiss IC, Feldon J (2001) *Psychopharmacology (Berl.)* 156: 305-26.
2. Geyer MA, Ellenbroek B (2003) *Prog Neuropsychopharmacol Biol Psychiatry* 27: 1071-9.
3. Weiss IC, Di Iorio L, Feldon J, Domeney AM (2000) *Behav Neurosci* 114: 364-73.

**PROMOTING CONTINUING EDUCATION IN PHARMACOVIGILANCE:  
THE EXPERIENCE OF “PHARMASEARCH”, A NETWORK OF GENERAL  
PRACTITIONERS REPORTING ADVERSE DRUG REACTIONS.**

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Underreporting of Adverse Drug Reactions (ADRs) is a major problem for all pharmacovigilance systems. Underreporting may be linked to lack of information and feedback provided to reporters. Given the positive results obtained in Italy and other countries, a network of Italian general practitioners (GPs) was set up in collaboration with the Italian Society of General Practitioners (SIMG) under the coordination of the Institute of Pharmacology of Messina University (Coordinating Centre, CC) to improve ADRs spontaneous reporting among GPs and to provide them with continuing education on drug safety and rational use of medicines.

The network of reporting GPs, called “Pharmasearch”, was set up in 2002 as collaboration between the CC and the SIMG. A group of GPs voluntarily forward to the CC a copy of each ADR reporting form filed in. The CC, after receiving the form, provides reporters with a personalised comment to the ADR reported, according to data retrieved from international literature. Moreover, GPs periodically receive reports about the activities of the network, including regular updates illustrating ADRs received, drugs implicated, etc. All cases of interest are studied in depth and spread to all participating GPs. Furthermore, a periodic bulletin (Inform@rete) is sent to participants, describing topics and news coming from international literature and regulatory agencies. This, in order to sensitise GPs about the importance of reporting ADRs and to keep them updated on the safety profiles of drugs and their correct use. To achieve this goal, a specific section of the website [www.farmacovigilanza.org](http://www.farmacovigilanza.org) (SIMGxFarmacovigilanza) has also been created, containing case reports published on the basis of GPs reported ADRs.

Up to 30 Dec. 2006, the number of reporting GPs was 261 (out of a total of 360 participants). At the start, the number of reporters was low but quickly rose; but in the latest years it is evident a negative trend in comparison to the first years, despite a mild increase in 2006. Until 30 Apr. 2007, the total number of reports filed was 2596, thus the average number of reports sent per quarter was 162.25. Quinolones, tramadol, coxibs and statins were the drugs most frequently involved in ADRs.

Although a decrease of reports and reporting GPs has occurred, the activation of local centres seems to be efficacious in involving GPs more directly and, thus, in increasing the number of reporting physicians. The website [www.farmacovigilanza.org](http://www.farmacovigilanza.org) has proved to be a useful point of reference in providing GPs with in-depth and up-to-date information concerning the ADRs they have reported on and may encounter in the future. A forum of discussion dedicated to pharmacovigilance and appropriateness of drug prescription is planned.

**ALTERATIONS OF N/OFQ-NOP GENE EXPRESSION IN EXPERIMENTAL MODELS OF PARKINSON'S DISEASE****Cavina Chiara***Department of Pharmacology, University of Bologna, via Irnerio 48, 40126 Bologna  
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Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized by motor disturbances such as akinesia, bradykinesia and tremor, often accompanied by cognitive impairment and depression. Loss of dopamine (DA) neurons projecting from the *substantia nigra* (SN) to the striatum (CP) and related plastic changes in motor circuits underlie PD.

The neuropeptide nociceptin/orphanin FQ (N/OFQ) is the endogenous ligand for the opioid-like receptor ORL-1, recently referred to as NOP. This peptidergic system presents marked structural analogies with the three different opioid receptors MOP, KOP, DOP and their related peptides, nevertheless there is no cross-interaction between the neuropeptides and the receptors of the N/OFQ system and of the opioid system.

N/OFQ has been shown to be involved in a variety of functions such as learning and memory, locomotory activity, food consumption, neuronal excitability, rewarding mechanisms and nociceptive transmission; moreover, N/OFQ has been shown to cause akinesia by means of glutamate release in SN (1); furthermore administration of NOP antagonists contrast akinesia (2). In fact, the role of N/OFQ system has been recently demonstrated in model of PD also by our group (2).

In order to confirm the involvement of the opioid neuropeptides in PD, the aim of the present study was to investigate the influence of the dopaminergic system on nociceptin/orphanin FQ and its receptor gene expression regulation in selective rat brain areas.

We therefore observed the effect of 6-hydroxydopamine (6-OHDA) and methylphenylpyridinium (MPP+), dopaminergic neurotoxins causing denervation of dopaminergic neurons and used to induce PD experimental model (3), in rat SN and CP.

For our purpose, to better define the role of dopamine (DA) in the regulation of nociceptin/NOP gene expression, selective depletion of dopaminergic neurons was induced in rats by a single administration of 6-OHDA (40 µg/10 µl) and MPP+ (40 µg/10 µl) i.c.v. The motor activity in rats was evaluated for 10 days after administration; both neurotoxins induced a reduction of motor activity only the first day. Ten days after the treatment, tissues (SN and CP) were processed, total RNA was isolated using OMNizol and quantified by RT-PCR.

In the SN, the disruption of dopaminergic neurons produces a remarkable increase in nociceptin gene expression after administration of 6-OHDA (149.4±10.1% vs. control) and MPP+ (161.5±19.1% vs. control). In the CP, N/OFQ mRNA levels decrease significantly after both treatments (48.7±3.7% and 53.1±9.0 vs. controls). Moreover, we observed a decrease of NOP gene expression after administration of 6-OHDA and MPP+ in the SN (72.4±5.6% and 85.9±5.1% vs. controls) and in the CP (47.9±10.1% and 46.5±7.2 vs. controls).

These results might suggest that DA modulates N/OFQ-NOP system gene expression in SN and CP, strengthening the hypothesis that the neuropeptidergic system could be strongly involved in the mechanisms underlying Parkinson's disease. Furthermore, data obtained may suggest a potential employment of NOP receptor antagonists to attenuate symptoms correlated to the degeneration of nigrostriatal dopaminergic pathway.

## References:

1. Marti M, Guerrini R, Beani L, Bianchi C, Morari M (2002) Neuroscience 112: 153-60.
2. Marti M, et al. (2005) J Neurosci 25: 9591-601.
3. Kahle PJ, Haass C, Kretschmar HA, Neumann M (2002) J Neurochem 82: 449-57.

## IN VITRO ANTICANCER ACTIVITY OF ECHINACEA EXTRACTS, FRACTIONS AND ISOLATED COMPOUNDS ON HUMAN CANCER CELL LINES

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Nine species of *Echinacea* genus are known and only three are currently used in therapy: *E. angustifolia*, *E. purpurea* and *E. pallida*. Several classes of active constituents have been identified in these species, including caffeic acid derivatives, alkylamides, polyacetylenes, and polysaccharides. These compounds are responsible for the antiinflammatory, immunostimulatory, antioxidant and cicatrising activities of this plant (1). *Echinacea* is one of the best selling herbal medicines in the United States and Europe (2), and its use is very popular among cancer patients mainly for its immunomodulatory activity (3) due to high molecular weight polysaccharides (arabinogalactans) and alkylamides (4).

This project research has been focusing on the potential *in vitro* anticancer activity of the medicinal *Echinacea* species hexanic extracts, fractions and isolated compounds on human cancer cell lines.

In the first part of the project, the effect of total root hexanic extracts derived from the three medicinal *Echinacea* species was evaluated on the viability of human pancreatic, MIA PaCa-2, and colonic, COLO320, cancer cell lines. All the three species induced an antiproliferative activity and *E. pallida* resulted to be the most active one, showing a concentration (30-300 µg/mL) and time (4-72 hours) dependent effect. Moreover, the cell death mechanism involved in *E. pallida* anticancer effect was investigated, finding an increase in caspase 3/7 activity and in cytoplasmatic internucleosomal DNA fragments, both related to the apoptotic process (5). The different antiproliferative effect among the *Echinacea* species was in agreement to the different phytochemical profile of the *E. pallida* hexanic extract, rich of polyacetylenes, which are almost absent in the other two species (containing mainly alkylamides) (6).

In the second part of the project, the cytotoxic effects of fractions and polyacetylenes isolated by a bioassay guided fractionation from the *E. pallida* hexanic extract and their potential bioavailability were investigated. Cell viability was evaluated by the colorimetric WST-1 assay and expressed as % of control (cell viability under vehicle treatment). Apoptotic cell death was assessed by an immunoenzymatic assay revealing the cytosolic internucleosomal DNA enrichment and by the evaluation of caspase 3/7 activity. Bioavailability studies were carried out using the Caco-2 cell monolayer, an *in vitro* model of intestinal permeability (7),

All the polyacetylenes exhibited a concentration- and time-dependent cytotoxicity (range 0.1-100 µM for 24-72h) on both cell lines and a greater potency on colonic cancer cells. Apoptotic cell death was demonstrated to be involved in the cytotoxic effect of the most active polyacetylene, as revealed by both the assays used. Finally, the polyacetylenes were found to cross the Caco-2 monolayer suggesting a likely bioavailability when taken orally.

### References:

1. Speroni E. et al. (2002) J Ethnopharmacol: 265-72
2. Chen Y. et al. (2005) J Nat Prod: 773-6
3. Richardson M.A. et al. (2000) J Clin Oncol : 2505-14
4. Miller S.C. (2005) Evid Based Complement Alternat Med: 309-314
5. Chicca A. et al. (2007) J Ethnopharmacol: 148-53
6. Barnes J. et al. (2005) J Pharm Pharmacol : 929-54
7. Matthias A. et al. (2004) J Clin Pharm Ther : 7-13

**SELECTIVE ANTAGONISM OF ADENOSINE A<sub>2A</sub> RECEPTOR ON MOTOR BEHAVIOR AND NEURONAL NITRIC OXIDE SYNTASE EXPRESSION IN AN ANIMAL MODEL OF HUNTINGTON DISEASE**

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Huntington disease (HD) is an autosomal, dominantly inherited neurodegenerative disorder characterized by progressive motor and cognitive disturbances caused by an expansion in CAG repeats in the IT15 gene, which encodes the Huntington protein. A pathogenetic role for excitotoxic cell death mediated by increased glutamatergic excitotoxicity in the striatum has been proposed. Drugs able to modulate striatal levels of glutamate are thus candidates to protect striatal neurons from neurodegeneration. Extracellular concentration of adenosine increases in the striatum of HD transgenic R6/2 mice in symptomatic phase (10-11 weeks) and the selective antagonist of adenosine A<sub>2A</sub> receptors, SCH 58261, directly administered in the striatum, significantly reduces glutamate outflow 1. In this work we investigated the effect of SCH 58261 chronically administered (0.01 mg/kg i.p. twice a day for two weeks) on body weight, motor deficit and neuronal nitric oxide synthase (nNOS) protein striatal expression in HD (R6/2) mice (n=7) at 10-11 weeks of age. SCH 58261 did not modify the body weight and did not affect rotarod test. The drug tended to improve the inclined plane test in comparison to non-treated mice (n=6-8).

Double immunostaining studies by antibodies against the c-fos early gene protein and against neurons showed that c-fos protein was not changed in the brain of transgenic mice (n=5) in comparison to wild type mice (n=5) at 10 weeks of age, but it was clearly incremented in the piriform cortex in 14 weeks-old mice (n=2). The increase in c-fos expression indicates late cell activation limited to the cortex.

Immunoistochemistry studies with antibodies against nNOS protein showed that SCH 58261 increased nNOS protein expression (about 55%) in the striatum of HD treated mice at 11 weeks of age (n=6) in comparison to wild-type mice (n=5). SCH 58261 subchronically administered (three doses in 24 h, 0.01 mg/kg i.p.), significantly increased nNOS expression (about 24%) in the striatum (n=3) in comparison to vehicle-treated mice (n=2). Since in R6/2 mice treated by NOS inhibitors and R6/2 mice missing both copies of the nNOS3 there was an acceleration in disease progression, an increase in nNOS expression may represent a possible therapeutic strategy in HD.

References:

1. Gianfriddo M, Melani A, Giovannini MG, Pedata F (2004) *Neurobiol Dis* 17: 77-88.
2. Deckel AW, et al. (2001) *Brain Res* 919: 70-81.
3. Deckel AW, Tang V, Nuttal D, Gary K, Elder R (2002) *Brain Res* 939: 76-86.

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## NITRIC OXIDE MODULATES DOPAMINE TRANSMISSION IN THE PREFRONTAL CORTEX OF AN ANIMAL MODEL OF SCHIZOPHRENIA

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Schizophrenia is a severe mental disorder characterized by thought disturbance, abnormal perception, impaired cognition, bizarre behaviour and social isolation (1).

One of the so called “environmental model” of schizophrenia is the isolation rearing of rats (2), which provides a non pharmacological and developmental specific method of inducing schizophrenic-like deficits (3). One of the many candidates found to be linked to schizophrenia is nitric oxide (NO). Functionally, NO may act as a hormone, neurotransmitter, paracrine messenger, mediator, cytoprotective and cytotoxic molecule. NO has multiple cellular molecular targets and it has been demonstrated that NO modulates the release of dopamine (DA), glutamate, and  $\gamma$ -aminobutyric acid (GABA) in various brain regions (4-5).

The aim of the present study was to characterize the *in vivo* local functional relationship between nitrenergic, glutamatergic, and GABAergic systems, and the nature of the neuromodulatory control of NO on dopaminergic transmission in the prefrontal cortex of socially isolated (ISO) or group-housed (GRP) Wistar rats.

*In vivo* microdialysis sampling showed that basal levels of DA and its metabolites, homovanillic acid (HVA) and 3,4-dihydroxy phenylacetic acid (DOPAC), of GRP rats compared to ISO rats were not different. Neither SNAP (1 mM, 20 min), bicuculline, a GABA<sub>A</sub> receptor antagonist (50  $\mu$ M, for 100 min and during SNAP challenge) nor memantine, a NMDA receptor antagonist (100  $\mu$ M, for 100 min and during SNAP challenge) affected DA levels during drug administration in GRP and ISO rats. However, extracellular DA concentrations were increased after co-administration of SNAP and bicuculline. The increase was still significant 3 h after treatment and was observed in isolation-reared and in socially reared rats.

The levels of DA metabolites were not affected by SNAP in ISO and GRP, but increased 1 h after bicuculline perfusion only in ISO rats.

In conclusion, these results suggest a different NO-induced neuromodulation of dopaminergic transmission in the prefrontal cortex of freely moving rats and show that endogenous GABA and glutamate control the activity of dopaminergic transmission through NO pathway.

### References:

1. Bernstein HG, Bogerts B, Keilhoff G (2005) Schizophr Res 78: 69-86.
2. Weiss IC, Feldon J (2001) Psychopharmacol (Berl) 156: 305-26.
3. Geyer MA, Ellenbroek B (2003) Prog Neuropsychopharmacol Biol Psychiatry 27: 1071-9.
4. Iravani MM, Millar J, Kruk ZL (1998) J Neurochem 71: 1969-77.
5. Trabace L, Kendrick KM (2000) J Neurochem 75: 1664-74.

## DIFFERENT STRATEGIES TO MODULATE ENDOCANNABINOID SYSTEM IN ORDER TO TREAT NEUROPATHIC PAIN

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The findings that the endocannabinoid system has an important role in the modulation of pain provided a new therapeutical possibility in the management of a debilitating type of chronic pain, the neuropathic one, refractory to common analgesics. Although CB1 agonists have long been considered as potential drugs, these agents produce substantial psychotropic effects that limited their clinical utility. In this regard, inhibitors of endocannabinoid metabolic enzymes or cellular transport, represent an alternative, since these agents act upregulating the endocannabinoid tone only at the location where their synthesis has been stimulated, without causing significant side effects. URB597, a FAAH inhibitor, and VDM11, a selective inhibitor of endocannabinoid transport, were compared in a widely used animal model of neuropathic pain, the chronic constriction injury of the rat sciatic nerve (CCI). Both drugs have been daily administered to rats for 7 days starting from the 7<sup>th</sup> day after the surgical procedure. The repeated administration of URB597 (10 mg/kg i.p.) led to a partial antihyperalgesic effect, while the repeated administration of VDM11 (10 mg/kg s.c.) resulted in a total relief of thermal hyperalgesia (assessed by plantar test, 24 hours after the last administration), already at 4 days after the beginning of treatment, suggesting a major efficacy of the endocannabinoid transport inhibitor. Since antagonism studies revealed that the antihyperalgesic effect evoked by both compounds was mediated by CB1 receptors, further studies are in progress to ascertain the presence/absence of psychoactive effects. At the light of the cross talking between endocannabinoid and endovanilloid system and the key role of the vanilloid receptor TRPV1, a nonselective cation ion channel, in nociception (it is expressed in primary afferent nociceptive neurons), molecules sharing endocannabinoid and vanilloid properties might represent novel tools of therapeutical significance. In this sense, arvanil (2 mg/kg i.p.), a “hybrid” ligand compound with capability to block the endocannabinoid transporter and to inhibit FAAH and that also possesses direct agonist activity at the cannabinoid CB1 and vanilloid TRPV1 receptors, was chronically administered to CCI rats following the same experimental procedure previously described. Arvanil was able to counteract neuropathic pain behaviour abolishing thermal hyperalgesia. The antihyperalgesic effect was completely reversed by capsazepine, a selective TRPV1 antagonist, suggesting that this compound acts only through TRPV1 receptors. Since it has been demonstrated that the expression of TRPV1 receptors is dramatically increased under inflammatory conditions, the pattern of expression of TRPV1 has been characterized in the injured sciatic nerve, showing an increased expression of TRPV1 receptors in unhealthy animals (measured by immunohistochemical, western immunoblotting and RT-PCR techniques), strongly supporting the TRPV1 as new target in pain modulation. In conclusion, all together these findings suggested that the different modulation in the endocannabinoid tone could represent an innovative approach in pain relief.



## DENDRIMERIC NEUROTENSIN-DERIVED PEPTIDES CONJUGATED TO FLUORESCENT OR RADIODIAGNOSTIC PROBES TO BUILD UP SPECIFIC PERSONALIZED THERAPIES

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Specific delivery is one of the most required goals to develop non-toxic cancer drugs. Recently, it has been demonstrated that solid tumours often overexpress receptors for hormone-peptides such as somatostatin, neurotensin (NT) and bombesin (1). NT receptors are overexpressed in adenoma colon cancer cell lines (HT29) and are an useful tool to develop specific tumour targeting. In a previous work we demonstrated that peptides in a tetrameric form resist to serum protease and are still able to bind their receptors (2-3). By knowing this, we synthesized NT branched peptides in order to develop specific and protease resistant drugs.

We focused on the conjugation of 8-13 NT-like peptides with fluorescein and studied the localization of NT-receptor-bound peptides in tissue slices from tumour transplanted nude mice with confocal microscopy.

Synthesis of 8-13 NT (monomer sequence: RRPYIL) tetra-branched peptides was obtained by solid phase synthesis with Fmoc-strategy. Synthesis was done on TGR resin. Amino acid coupling was carried on by preactivation of carboxylic groups using HBTU/DIPEA method. Complete tetrameric peptides were conjugated with 5-carboxy fluoresceine including an hydrophilic (PEG) spacer between peptide and the fluorophore. Peptides were then cleaved from resin and fully deprotected with TFA 95%. Peptides were purified with reverse phase HPLC (C18 column and 80-20% water/methanol, 30 min gradient). Peptides were then checked with MalDI-ToF mass analysis.

**Ex-vivo immunofluorescence**

An HT29 solid tumour was removed from transplanted nude mice, frozen in tissue-tek and sectioned with a cryostat (15 µm sections). The sections were incubated for 1 h at 37°C with NT4(8-13)-fluorescein (5 µg/ml) or with an unrelated tetrabrached-fluorescein (5 µg/ml). Images taken with confocal microscope were analyzed and compared with a software that measured pixel. Fluorescent signal was observed with 488 nm laser and 500 nm of emission.

We obtained a multimeric NT peptide conjugated with fluorescein (NT4-Fluo). Images quantification from *ex-vivo* immunofluorescence showed significant difference of peptide binding to its specific receptor measured as difference of signal between NT peptide and a scramble peptide both conjugated with fluorescein.

NT receptors were uniformly expressed in the solid tumours and could be suitable targets for the diagnostic use of dendrimeric NT peptides conjugated to fluorescent or radiodiagnostic probes in order to build up specific personalized therapies.

References:

1. Reubi JC (1997) Q J Nucl Med 41: 63-70.
2. Bracci L, et al. (2003) J Biol Chem 278: 46590-5.
3. Falciani C, et al. (2007) Chem Biol Drug Des 69: 216-21.

## DEXAMETHASONE HYALURONAN ESTERS AS NOVEL BIOCONJUGATES IN LEUKEMIA'S PHARMACOLOGICAL TREATMENT

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Cytotoxic drugs cannot discriminate between normal and tumour cells, and they generally hit cells in active replication. The goal of actual research is to increase selectivity of cytotoxics, to improve efficacy of the therapy, minimizing their side effects.

One promising molecule to be used as a vehicle for anticancer drugs is hyaluronic acid (HA). This polymer, constitutively found in extracellular matrix, can bind the CD44 receptor that results overexpressed in tumour cells. HA alone, moreover, shows a promising activity as an antiangiogenic compound, its potency and type of action depending on molecular weight, and increasing data suggest HA is also capable to affect malignant cell ability to invade tissues.

The aim of the present study was to evaluate the efficacy of different bioconjugates containing HA and dexamethasone alone (named D1 and D3), and HA, dexamethasone and butyric acid (named BD1 and BD2) against the growth of two leukaemia cell lines, NB4 (human acute promyelocytic leukaemia) and P388 (murine leukaemia), and against a pancreatic carcinoma cell line, MIA PaCa2. Tests were performed both *in vitro* and *in vivo*.

Initially, the three lines were screened for the expression rate of CD44 and for the cytotoxic response to the bioconjugates and to HA alone, using MTT test, comparing their effect to dexamethasone alone. The differentiate-inducing ability of the bioconjugates D3 and BD2, and of dexamethasone was also evaluated on NB4 line, using the NBT test. Drug interaction with CD44 and its relationship with the efficacy of bioconjugates were verified by using a CD44-blocking antibody on the human-derived cell lines.

An invasion test with transwell plates covered with matrigel (in order to mime the extracellular matrix situation) was performed on MIA PaCa2 cell line and HAs of different weight (from 8 to 200 kDa), whereas HA ability to affect angiogenic process, both as free polymer and as bioconjugate (D3 and BD2 compounds were used) was investigated using a commercial kit (Angiokit -TCS CellWorks).

Finally, tolerability and efficacy of dexamethasone, D3 and BD2 on Scid mice bearing human acute lymphoblastic leukaemia NB4 were checked.

All three lines expressed CD44. The cytotoxic effect of bioconjugates varied depending on the cell line, P388 showing the lowest response, NB4 the highest. Cytotoxic effect of the bioconjugates was decreased when an antibody against CD44 was used, indicating CD44-mediated internalization was the most used (but not the only) way by which the bioconjugates enter the cell. D3 and dexamethasone alone were almost comparable in our *in vitro* test, while BD3 showed a significant increase of activity when compared to dexamethasone alone, proving the efficacy of the association of the two drugs. As expected, HA of different mass weight alone showed no cytotoxic effect at the concentration used as bioconjugate.

D3, BD2, and dexamethasone were capable of inducing NB4 differentiation, D3 being the weakest, dexamethasone the strongest.

High molecular weight HA (200 kDa) resulted effective in decreasing MIA PaCa 2 invasion through Matrigel layer. A decrease of angiogenesis was detected both with High molecular weight HA (160 kDa) and D3 and BD2 bioconjugates, while no significative effect was noted for HA of medium molecular weight, as expected.

*In vivo*, dexamethasone alone showed no activity, dexamethasone treated mice sharing the same course of control non-treated mice, while a good response and a prolongation of life-expectancy was achieved using both D3 and BD2 conjugates ( $P < 0.01$ ).

These results indicate that HA bioconjugates D3 and BD2 are an innovative pro-drug delivery system of dexamethasone to tumour cells, and that their uptake is mediated by CD44 receptor. In particular, our *in vivo* results indicate that these compounds could be promising antineoplastic agents for the treatment of acute promyelocytic leukaemia.

## ANTHOCYANIDINS MODULATE THE CYTOTOXICITY OF IRINOTECAN AND OXALIPLATIN IN HUMAN COLON ADENOCARCINOMA CELLS

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Colon cancer is the second leading cause of cancer death in most western countries; 40 to 50% of patients who undergo potentially curative surgery alone ultimately relapse and die of metastatic disease. Chemotherapy is used as a first-line treatment for metastatic colorectal cancer to destroy cancer cells that have spread; the new agents incorporated into frontline therapies, irinotecan and oxaliplatin, have improved the prognosis, but still, drug resistance is the major cause of chemotherapy failure due to overexpression of ABC efflux pumps and glutathione-S-transferase. Flavonoids, the most common and widely distributed group of polyphenols in plants, show strong antioxidant activities and inhibitory effect on the growth of some cancer cells; these agents interact with ABC transporters and reduce the expression of GSTP in tumour cells and therefore are of interest for increasing the efficacy of antitumour agents. The aim of this study was to evaluate the cytotoxicity of anticancer agents irinotecan and oxaliplatin alone or in combination with anthocyanidins, a class of flavonoids widespread in fruits and vegetables, and to study their apoptotic effect in LoVo human colon adenocarcinoma cells. Cytotoxicity assay (MTT test) was performed to determine IC<sub>50</sub> of anticancer agents and anthocyanidins and to evaluate the effect of their combination in LoVo cells. To determine apoptotic effect we analysed DNA integrity by agarose electrophoresis and changes in nuclear morphology using a fluorescence microscope. IC<sub>50</sub> for the antitumour drugs was respectively 37.0 ±5.3 nM for camptothecin, 876.2±245.5 nM for oxaliplatin, 2.5±0.5 µM for SN-38 (the active metabolite of irinotecan), and 130.5±10.9 µM for irinotecan. IC<sub>50</sub> for anthocyanidins was: 37.6±3.3 µM (delphinidin), 46.9±1.8 µM (cyanidin), and 80.5±9.8 µM (malvidin). IC<sub>50</sub> for pelargonidin was not determined since it did not show any cytotoxic effect. The effect of the combination of anthocyanidins and antineoplastic drugs was also studied. Delphinidin (25 µM) significantly increased the cytotoxic effect of camptothecin 0.01 µM (P<0.001), oxaliplatin 0.1 µM (P<0.001) and SN-38 0.5 µM (P<0.01). The same concentration of cyanidin had similar effect in combination with camptothecin (P<0.001) but 50 µM cyanidin significantly increased cytotoxicity of all three drugs (P<0.001). Camptothecin (250 nM and 1 µM) induced nucleosomal DNA fragmentation typical of apoptosis in HL-60 cells (human promyelocytic leukemia cells) but not in LoVo cells. On the other hand, apoptotic cells with fragmented nuclei were observed in both HL-60 and LoVo cell lines after treatment with camptothecin (250 nM) and delphinidin (100 and 200 µM). Our results show that anthocyanidins, in particular delphinidin and cyanidin, exert an important cytotoxic action in the LoVo colon cancer cell line and increase the cytotoxicity of oxaliplatin, camptothecin and SN-38. Studies are in progress to clarify the mechanisms of this cytotoxic action such as detection of apoptosis, GSH level measurements and expression of drug transport proteins in cells treated with different concentrations of the antineoplastic drugs and flavonoids.

### References:

1. Arnould S. et al. Contribution of Apoptosis in the Cytotoxicity of the Oxaliplatin-Irinotecan Combination in the HT29 Human Colon Adenocarcinoma Cell Line. *Biochem Pharm* 64: 1215-1226, 2002
2. Germann UA. P-glycoprotein- A mediator of Multidrug Resistance in Tumor Cells. *Eur J Cancer* 32A, 6: 927-944, 1996
3. Linn SC., Giaccone G. MDR1/P-glycoprotein Expression in Colorectal Cancer. *Eur J Cancer* 31A, 7/8: 1291-1294, 1995
4. Hou DX. et al. Molecular Mechanisms Behind the Chemopreventive Effects of Anthocyanidins. *5(2004):321-325, 2004*
5. Hou DX. et al. Anthocyanidins Inhibit Activator Protein 1 Activity and Cell Transformation: Structure-Activity relationship and Molecular Mechanisms. *Carcinogenesis* 25(1): 29-36, 2004

## **OLANZAPINE EFFECTS ON A MODEL OF INSULIN-RESISTANCE INDUCED BY THE EXPOSURE TO A HIGH FAT DIET IN RATS**

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Type 2 diabetes mellitus (DM2) is one of the most common endocrine diseases worldwide. Moreover, some forms of psychopathology such as bipolar disorder, depression and schizophrenia show a DM2 prevalence significantly higher compared to that of general population. However, the increased risk of developing DM2 for psychotic patients became a health-emergency only with widespread use of new generation antipsychotics drugs, such as olanzapine. Clinical studies show an association between the therapeutic response and the metabolic alterations during antipsychotic therapy.

The aim of this study was to investigate olanzapine effects on a model of diet-induced insulin resistance in rats. The model is based on long-term consumption of a high-fat and high-carbohydrate diet (HF). As indicators of a condition of insulin resistance we selected: 1) blood glucose and plasma insulin levels at baseline, after a 16-18 h fast and after oral glucose load; 2) levels of expression of insulin receptor and its phosphorylated form in liver after insulin challenge in 16 h fasted rats by immunoblotting. Rats fed the HF diet were divided into 2 groups, one receiving saline and the other olanzapine (0.25 mg/kg/die i.p.) for 8 weeks. Two groups of rats fed the standard diet were divided into 2 groups, one receiving saline and the other olanzapine (0.25 mg/kg/die i.p.) for 8 weeks, i.e. olanzapine treatment and exposure to the hypercaloric diet began simultaneously. During the 8-weeks treatment, we recorded weekly body weight and food intake. We also detected rectal temperature before and after injection of saline or olanzapine at week 4 and 8 of treatment and we found a transitory condition of hypothermia in rats treated with olanzapine lasting for several h after each treatment.

At the end of the experiment, blood glucose and plasma insulin levels were detected in basal conditions, after a 16-18 h fast, and after oral glucose load. Two h after glucose load glycaemia was significantly increased in control and olanzapine rats fed the HF diet compared to control and olanzapine rats fed the standard diet. Two days later, following a 16 h fast, rats received insulin (2 IU/kg i.p.) and were sacrificed 10 min later. We measured the weight of liver, epididymal fat and gastrocnemius muscle; the rats fed with HF diet had the body weight and epididymal fat weight significantly increased compared to rats fed with standard diet. Moreover, the levels of total insulin receptor and of its phosphorylated form were decreased in rats fed the HF diet and the expression of UCP-1 (a protein implicated in non-shivering thermoregulation) was increased in rats treated with olanzapine.

## OSTEOGENIC DIFFERENTIATION OF HUMAN ADIPOSE-DERIVED STEM CELLS (HASCs) AND THEIR INTERACTIONS WITH SCAFFOLDS SUITABLE FOR SKELETAL TISSUE REGENERATION

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Adipose tissue can be harvested in large amounts with minimal morbidity. It contains numerous cells types, including human adipose-derived stem cells (hASCs) that have the ability to differentiate into several lineages, such as fat, bone, cartilage, muscle, endothelium, hepatocytes, and neuronal cells. The combination of this high differentiation potential and the large number of cells obtained from fat, suggests that this tissue can be a useful tool in biotechnology and in regenerative medicine. In this study, after characterization of undifferentiated hASC, we have analyzed their differentiation potential into osteogenic, chondrogenic, and adipogenic lineages. Moreover, cells-scaffolds interactions have been examined, in order to investigate the hASC potential for further orthopaedic and dental applications. hASCs were enzymatically isolated from lipoaspirate of 23 adult human donors (1), under informed consensus. Cells were expanded in culture by plastic adherence and then characterized by cytofluorimetric analysis, assaying the presence of MSCs-related cell surface antigens (2). hASCs were then induced to differentiate into osteogenic, chondrogenic, and adipogenic lineages, studying the best inducer supplement combinations, particularly for osteogenic differentiation (3). Changes in cell morphology, the expression of tissue-specific protein, like alkaline phosphatase and osteopontin, and ECM deposition were determined. We have also analyzed the interactions between differentiated hASC and several scaffolds like HAP, alginate, deproteinized bovine bone, human bone, and titanium. We obtained a cell yield of about  $5 \times 10^5$  cells/ml of raw lipoaspirates, with a constant proliferative trend and a clonogenic capacity varying from 10 to 20%, depending on the time in culture. Undifferentiated hASC were CD13+, CD105+, CD90+, CD29+, CD44+, CD49d+, CD45-, and CD71- (4), whereas osteogenic differentiation for two weeks increased CD29, CD49d and CD105 expressions without any effect on CD13 and CD90 expression. hASC differentiated into adipogenic and chondrogenic lineages and were steadily induced into osteoblasts when cultured in 10 nM dexamethasone and 150  $\mu$ M ascorbic acid, as showed by alkaline phosphatase increase and up-regulation of OPN expression already after two weeks. hASC seeded on suitable scaffolds were able to deposit tissue specific ECM, as assessed by SEM analysis and specific hystochemistry staining. In particular, we obtained very high level of calcium deposition culturing hASC on porous ceramic scaffold and on natural human bone and deproteneized bovine bone. No cytotoxic effects were observed and hASC proliferation was maintained. hASC may be a suitable cells source for tissue engineering, above all in orthopedic and dental fields. Furthermore, due to their abundance and differentiation capability, hASC can be used for screening of scaffolds biocompatibility and cell adhesion, before *in vivo* model application, and for testing drugs affecting the skeletal system.

### References:

1. Zuk P, et al. (2002) Mol Biol Cell 13: 4279-95.
2. Awad HA, Wickham MQ, Leddy HA, Gimble JM, Guilak F (2004) Biomaterials 25: 3211-22.
3. de Girolamo L, Sartori MF, Albisetti W, Brini AT (2007) J Tissue Eng Reg Med 1: 154-7.
4. Albisetti W, de Girolamo L, Parafioriti A, Rovati LC, Brini AT (2005) Archivio di Ortopedia e Reumatologia 116: 19-20.

**THE ROLE OF SPHINGOSINE KINASE/SPHINGOSINE-1-PHOSPHATE PATHWAY IN INFLAMMATORY RESPONSES****De Gruttola Luana**

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Sphingosine-1-phosphate (S1P) is a product of sphingolipidic metabolism following the sequential activation of sphingomyelinase, ceramidase, sphingosine kinase (SphK). S1P regulates numerous biological functions. To date, eight S1P receptors have been identified denominated EDG, but recently renamed S1P (1-2). Alternatively, S1P can act as intracellular messenger and regulate Ca<sup>2+</sup> intracellular levels (3). Recently S1P has been proposed as a new inflammatory mediator. In particular, it has been show that S1P receptor and SphK are widely expressed by cells involved in inflammatory processes such as macrophages, neutrophils, monocytes and lymphocytes. Aim of this study was to assess the role of S1P and SphK in the development of inflammatory responses by using inflammation model *in vivo*, such as paw edema and air pouch.

Mice were divided in 3 different groups and treated with vehicle, or DTD, a SphK inhibitor or L-cycloserine, a systemic S1P depletory. After 30 min, all mice received a subplantar injection of 50 µl of carrageenan 1%. Mouse paw edema is characterized by two different phases: the first reaches the plateau at 4 h resolving within 6 h; the second starts at 24 h reaching the plateau at 72 h. Paw volume was measured by using a hydroplethismometer specially modified for small volumes immediately before the subplantar injection and 2, 4, 6, 24, 48, and 72 h thereafter. The increase in paw volume was evaluated as the difference between the paw volume at each time point and basal paw volume. Air pouches were developed by s.c. injection of 5 ml of sterile air into the back of mice. Three days later, 5 ml of sterile air was re-injected in the same cavity. After another 3 days (6 days after the first air injection), 1 ml of zymosan 1% w/v or vehicle (saline) was injected into air pouch. Mice were divided in 3 different groups treated with vehicle, DTD or L-cycloserine. At 4 h after zymosan injection, mice were killed and the exudates in the pouches were collected with 1 ml of saline and total cell count was evaluated by optical microscopy in the cell suspension diluted with Turk's solution.

Treatment of mice with DTD induces a significant inhibition of both phases of mouse paw edema. Similarly L-cycloserine displayed a significant reduction in paw edema development with a more marked effect on the first phase. (MPO) (myeloperoxidase) evaluation on paws harvested by mice of the three different groups confirms a significant reduction in neutrophil infiltration following treatment with both DTD and L-cycloserine. The involvement of SphK/S1P pathway in cell migration was confirmed by air pouch experiments, where a significant reduction in cell migration was observed following treatment with DTD and L-cycloserine.

## References:

1. Pyne S, Pyne N(2000) *Pharmacol Ther* 88: 115-31.
2. Chun J, et al. (2002) *Pharmacol Rev* 54: 265-9.
3. Ghosh TK, Bian J, Gill DL (1994) *J Biol Chem* 269: 22628-35.

## CRH INHIBITS CELL GROWTH OF HUMAN NEUROBLASTOMA CELL LINE IMR-32 AND MEDULLOBLASTOMA CELL LINE DAOY

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The hypothalamic peptide corticotrophin releasing hormone (CRH) plays an important role in the regulation of hypotalamo-pituitary-adrenal axis and acts to integrate endocrine, autonomic and behavioural responses to stressors.

The effects of CRH on target tissues are mediated through the activation of two high affinity membrane receptors: CRH type 1 and type 2 (CRH-R1 and CRH-R2), which belong to the family of seven transmembrane domain receptors coupled to G protein and activating adenilate cyclase. These receptors display different distribution within several tissues, reflecting distinct biological functions.

The expression of CRH receptors, both CRHR-1 and CRHR-2 types, in human cancers originating from tissues that may or may not express CRH receptors has been reported. Moreover, various human cancer cell lines, such as endometrial adenocarcinoma, breast cancer cells, neuroblastoma, small cell lung cancer and melanoma lines also express CRH receptors. These findings suggest that CRH may play a role in the control of neoplastic cell growth. We have previously shown that CRH is able to inhibit the proliferation of a human endometrial adenocarcinoma cell line, Ishikawa (IK) cells, as well as of MCF-7 human breast cancer cells but, to date, there is little information on the ability of CRH to inhibit cell proliferation in cell lines derived from other tumour types. Therefore we investigated the possible antiproliferative effects of CRH on cell lines derived from the nervous system, e.g. the human neuroblastoma cell line IMR 32, which expresses functional CRH-R1 receptors, and the medulloblastoma cell line DAOY.

IMR 32 and DAOY cells were cultured in Eagle's minimum essential medium with Earle's salts supplemented with 10% fetal bovine serum, 2 mM L-glutamine, penicillin-streptomycin, Na-pyruvate, and non essential aminoacids. Cells were maintained at 37°C in a 5% CO<sub>2</sub> and 95% O<sub>2</sub> humidified atmosphere. IMR32 cells were plated in 60 mm dishes at a density of 2x10<sup>5</sup> cells/dish, whilst DAOY cell line was plated at a density of 8x10<sup>4</sup> cells/dish.

The treatment of IMR32 cells with CRH induced a time- and concentration-dependent inhibition of cell growth, with a maximal effects (47±3% inhibition, P<0.01 vs. control) after 4 days at the dose of 100 nM. This effect was counteracted in a concentration-dependent manner by the CRH receptor antagonist astressin. The measurement of CRH-induced intracellular cAMP accumulation suggested the downstream involvement of cAMP-PKA pathway in the effect of CRH in IMR32 cell growth. CRH (100 nM) also decreased significantly the proliferation of DAOY cells (39±3% inhibition, P<0.05 vs. control) after 4 days. These results suggest that CRH may play a role in the control of cell growth in CNS tumours; currently ongoing studies are testing the hypothesis that such effect of CRH is mediated via the activation of CRH-R1 receptors.

### References:

1. Graziani G, Tentori L, Muzi A, Vergati M, Tringali G, Pozzoli G, Navarra P. *Moll Cell Endocrinol* 264:44-49, 2007.
2. Graziani G, Tentori L, Portarena I, Barbarino M, Tringali G, Pozzoli G, Navarra P. *Endocrinology* 143: 807-813, 2002.
3. Graziani G, Ferrandina G, Pozzoli G, Vergati M, Muzi A, Legge F, Tentori L, Scambia G, Navarra P. *Oncol Rep.* 15: 375-379, 2006.

**EFFECTS OF DP7 ON HUMAN AND RAT LIVER CYP-DEPENDENT ACTIVITIES: A KINETIC STUDY****D'Elia Paolo***Dipartimento di Scienze Biomediche, Università degli Studi di Siena, via Aldo Moro 2, 53100 Siena  
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Many cytotoxic agents and multidrug resistance (MDR) inhibitors are both substrates of permeability-glycoprotein (P-gp) and cytochrome P450 (CYP) 3A4. This results in unpredictable pharmacokinetic interactions. The ideal MDR-reverter should inhibit P-gp leaving CYP unaffected (1). Differently substituted 1,4-dihydropyridines were investigated for their activity as MDR reverters and the novel 3,5-dibenzoyl-4-(3-phenoxy-phenyl)-1,4-dihydro-2,6-dimethylpyridine (DP7) was found to be a powerful P-gp inhibitor, almost devoid of cardiovascular effects (2-3).

The aim of the present study was to investigate the effects of DP7 on CYP activities by human and rat liver microsomes. When rat microsomes were incubated with DP7, concentration-inhibition curves were obtained with use of selective substrates, markers of CYP activities. Ethoxy-resorufin (ETR) was used as substrate for CYP1A1, penthoxy-resorufin (PTR) for 2B, methoxy-resorufin (MTR) for 1A2 and benzyloxy-resorufin (BZR) for 1A1/2, 2B, 3A. DP7 inhibitions gave IC<sub>50</sub> values of 3.8 μM for PTR, 3.8 μM for ETR, and 10.4 μM for BZR and were not competitive in nature; moreover, they were reversible. DP7 inhibition of CYP3A enzyme activities of rat (3A2) and human (3A4) liver microsomes was assessed fluorimetrically using 7-benzyloxy-quinoline (BQ) and [3-[3(3,4-difluorobenzyl)oxy]-5,5-dimethyl-4-[4-(methylsulfonyl)phenyl]furan-2(5H)-one] (DFB). When BQ was used as substrate of rat microsomes, DP7 inhibited its oxidation with an IC<sub>50</sub> value of 4.17 μM, while this activity was inhibited by only 25% at the highest DP7 concentration used (75 μM) on human microsomes. On the contrary, when DFB was used as substrate, DP7 showed identical IC<sub>50</sub> values (34.7 μM) with microsomal preparations from either species.

BZR-O-dealkylase (BROD) activity of male rat liver microsomes was also investigated in presence of dicumarol; this compound was added to the reaction mixture in order to block the NADPH:quinone oxidoreductase 1. In agreement with data reported by Ninci and De Matteis (4), in presence of dicumarol BROD activity was activated in a concentration-response manner. This activation was species and gender specific for male rat (further experiments on human, rabbit, and female rat liver microsomes showed no activation).

The mechanism of this activation appeared to be of the mixed type. Inhibition by DP7 of BROD activity measured in presence of dicumarol was much stronger than in its absence (IC<sub>50</sub> value of 0.15 μM). The mechanism of inhibition was of the mixed type with K<sub>i</sub> and K<sub>i</sub>' values of 0.14 and 0.86 μM, respectively. Moreover, this inhibition was reversible, dependent neither on time nor on the formation of possible metabolites of DP7. These findings suggest that DP7 binds reversibly to CYP450, giving rise to sterical hindrance to the subsequent binding of both activator (dicumarol) and substrate (BZR).

In conclusion, the moderate inhibition of CYP isoforms in rat liver microsomes and the weak inhibition of human CYP3A4 enzyme activity operated by DP7, support the role of this compound as a lead for the development of novel MDR revertering dihydropyridines of therapeutic interest.

**References:**

1. Fusi F, et al. (2006) *Current Drug Targets* 7: 949-59.
2. Saponara S, et al. (2004) *Br J Pharmacol* 141: 415-22.
3. Saponara S, et al. (2007) *Eur. J. Pharmacol.* 563: 160-3.
4. Ninci R, De Matteis F (2005) *Pharmacologyonline* 3: 41-53.

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**CHARACTERIZATION OF MUCIN DEPLETED FOCI (MDF), NEW BIOMARKERS IN EXPERIMENTAL COLON CARCINOGENESIS****Femia Angelo Pietro***Dipartimento di Farmacologia Preclinica e Clinica "Mario Aiazzi Mancini", viale Pieraccini 6, 50139 Firenze**E-mail: angelo.pietro@unifi.it*

Colon carcinogenesis induced in rodents by azoxymethane (AOM) or 1,2-dimethylhydrazine (DMH) evolves through the sequential formation of histopathological lesions similar to those observed in human carcinogenesis. In this process, preneoplastic lesions closely related to tumours can be used as biomarkers in studies aimed at the identification of agents affecting colon carcinogenesis. Recently, we reported the identification of Mucin Depleted Foci (MDF), putative preneoplastic lesions in the colon of carcinogen-treated rats (1). A multiple approach was used to characterize these lesions and to test their usefulness as biomarkers in colon carcinogenesis. Molecular characterization was carried out studying: mutations in the *Apc* and *K-ras* genes, frequently mutated in human colon cancer (2), and the expression of two mucins (MUC2 and MUC5AC) altered in colon carcinogenesis (3-4). The correlation between MDF and tumours was tested in rats using a calorie-restricted (CR) diet (known to inhibit colon cancer) (5), and in mice, using a model of colonic inflammation, associated with an increased risk of developing colorectal cancer (6).

In rat studies, tumours, MDF and Aberrant Crypt Foci (ACF), putative preneoplastic lesions previously described in rodents and humans, were harvested from F344 rats treated with two injections of DMH (150 mg/kg s.c.). Laser microdissected paraffin sections of MDF, ACF, and tumours were used for DNA extraction. A nested-PCR was used to amplify *Apc* and *K-ras* gene segments, which were then sequenced. MUC2 and MUC5AC expression was evaluated by immunohistochemistry in paraffin sections of MDF, ACF and tumours. For the studies on the correlation with carcinogenesis, rats injected with DMH, were randomly divided in two groups and fed a control high-fat diet or a calorie-restricted (CR) diet (70% of the calories consumed by controls). In colonic inflammation model, CD1 male mice were treated with a subcarcinogenic dose of AOM (10 mg/kg i.p.) and one week later treated for 1 week with dextran sodium sulphate (DSS) (2% DSS in drinking water) to induce colitis. Controls received only water. Mice were sacrificed 6 weeks later and MDF determined. *Apc* mutations were observed in 25% (6/24) of MDF, in 0% (0/24) of ACF and in 30% (7/23) of tumours. The frequency of lesions with *K-ras* mutations was 23% (3/13) in MDF, 100% (14/14) in ACF and 5% (1/18) in tumours. MDF and tumours, but not ACF, showed a dramatic reduction in the expression of MUC2. The expression of MUC5AC was almost absent in normal mucosa, but was increased in all the lesions. Calorie restriction significantly reduced MDF (MDF/rat were  $6.5 \pm 2.4$  (SD) and  $3.6 \pm 1.9$  in controls and CR, respectively,  $P=0.01$ ), while colonic inflammation by DSS significantly increased the number of MDF/colon compared to controls (MDF/mouse were  $10.1 \pm 2.2$  (SD) and  $1.2 \pm 1.6$  in DSS and control, respectively,  $P=0.01$ ).

MDF show genotypic and phenotypic alterations similar to those observed in tumours. Moreover, MDF and tumour occurrence are correlated in different experimental setting. These results support the precancerous nature of MDF and their usefulness as biomarker for experimental colon carcinogenesis.

## References:

1. Caderni G, et al. (2003) *Cancer Res* 63: 2388-92.
2. Pretlow TP, Pretlow TG (2005) *Biochim Biophys Acta* 1756: 83-96.
3. Mizoshita T, et al. (2007) *Histol Histopathol* 22: 251-60.
4. Bara J, Forgue-Lafitte ME, Maurin N, Fléjou JF, Zimber A (2003) *Tumour Biol* 24: 109-15.
5. Reddy BS, Wang CX, Maruyama H (1987) *Cancer Res* 47: 1226-8.
6. Tanaka T (2003) *Cancer Sci* 94: 965-73.

## CARDIOVASCULAR EFFECTS OF DE-ALCOHOLATED RED WINE ON ISOLATED RAT HEART

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Epidemiological studies suggest that a moderate consumption of wine, particularly of red wine, reduces the incidence of developing coronary heart disease. This beneficial effect is mainly attributed to de-alcoholated extract, such as the occurrence of polyphenol compounds in red wine (1). The present study focuses on the cardiovascular effects of red wine polyphenols (RWPs) on isolated rat hearts perfused according to the method described by Langendorff (constant flow) (2).

Left ventricular pressure (LVP), coronary perfusion pressure (CPP), and surface electrocardiogram (ECG) were measured in paced hearts (5 Hz, 0.6 msec, 8 V). The ECG parameters measured were: PQ interval (the atrio-ventricular conduction time), QRS interval (the intraventricular conduction time), and QT interval (the duration of ventricular depolarisation and repolarisation, i.e. the action potential duration). RWPs, expressed as  $\mu\text{g}$  of gallic acid equivalents per ml, were freeze-dried under vacuum (100 mtorr) (3).

In the presence of 2.8  $\mu\text{g}/\text{ml}$  RWPs, all the ECG parameters did not change appreciably (PR=34.4 $\pm$ 2.7 msec, control; 33.5 $\pm$ 2.2 msec, RWPs; QT=67.6 $\pm$ 3.0 msec, control; 69.6 $\pm$ 3.4 msec, RWPs; QRS=13.3 $\pm$ 0.4 msec, control; 13.9 $\pm$ 0.4 msec, RWPs, n=6).

RWPs caused relaxation of coronary arteries (maximum decrease at 2.8  $\mu\text{g}/\text{ml}$  RWPs was 20% of control values) and induced a concentration-dependent negative inotropic effect.

Both N( $\omega$ )-nitro-L-arginine methyl ester (300  $\mu\text{M}$ ), a competitive inhibitor of endothelial NO synthase (eNOS), and wortmannin (100 nM), an irreversible inhibitor of phosphatidylinositol 3 (PI3)-kinase, prevented coronary dilation induced by RWPs, leaving unaltered the negative inotropic effect.

In conclusion, these results suggest that RWPs, at concentrations reached in human blood after intake of 100 ml of red wine (4), probably induce the redox-sensitive activation of the PI3-kinase/Akt pathway in coronary endothelial cells. This, in turn, causes phosphorylation of eNOS, resulting in an increased formation of NO. On the contrary the inotropic action seems not to be mediated by endothelium-derived NO. Further studies are required to elucidate the mechanism by which RWPs elicit negative inotropic effect as well as to determine whether other endothelial-derived mediators are involved. Finally, these results might explain, at least in part, the molecular mechanisms responsible for the prevention of cardiovascular diseases associated with a moderate consumption of red wine polyphenols.

### References:

1. Gronbaek M, et al. (2000) *Ann Intern Med* 133: 411-9.
2. Langendorff O (1895) *Pflügers Arch* 61: 291-332.
3. Dragoni S, Gee J, Bennett R, Valoti M, Sgaragli G (2006) *Br J Pharmacol* 147: 765-71.
4. Stoclet JC, et al. (2004) *Eur J Pharmacol* 500: 299-313.

**IRINOTECAN METRONOMIC CHEMOTHERAPY IN METASTATIC COLORECTAL CANCER PATIENTS: A PHARMACOKINETIC AND PHARMACODYNAMIC STUDY****Fioravanti Anna***Dipartimento di Medicina Interna, Facoltà di Medicina e Chirurgia, Università degli Studi di Pisa, via Roma 55, 56126 Pisa**E-mail: anna.fioravanti@libero.it*

Truly long-term, regular frequency, low-dose chemotherapy (metronomic/antiangiogenic chemotherapy) has been recently developed but is still uncommon in adult oncology. Metronomic irinotecan (CPT-11) clinical trials for treatment of cancer have not been reported and pharmacodynamic (PD) and pharmacokinetic (PK) investigations have not been yet performed. The experimental hypothesis to be tested is whether CPT-11 metronomic regimens are feasible and effective, investigating the pharmacokinetic of CPT-11 and SN-38 (the active metabolite), the modulation of pro- (VEGF) and anti-angiogenic factor (thrombospondin-1, TSP-1) expression and establishing possible significant relationships among clinical PK and PD parameters. Twenty consecutive patients (M/F, 11/9; age range 51-79 years) with a diagnosis of metastatic colorectal carcinoma, treated with previous palliative chemotherapy with fluoropyrimidines, oxaliplatin and irinotecan were studied. The doses of CPT-11 administered as 63-day protracted continuous infusion, with no interruption, for each group of patients were as follows: CPT-11 1.4 mg/m<sup>2</sup>/day (n=7), CPT-11 2.8 mg/m<sup>2</sup>/day (n=5), CPT-11 4.2 mg/m<sup>2</sup>/day (n=8). Toxicities were scored according to NCI criteria. Drug levels were examined by HPLC, whereas plasma levels of TSP-1 and VEGF were evaluated by ELISA. Four patients (20%) obtained a stable disease despite the tumour resistance to CPT-11. This stabilization of disease lasted a median period of 3.9 months (range, 3 to 5 months). In the remaining 16 patients (80%) a progression was observed. Toxicities  $\geq$  grade 1 were not observed. Three (15%) and five patients (25%), respectively, experienced a transitory grade 1 diarrhoea and grade 1 nausea, resolved without interrupting the treatment. Haematological toxicity was not observed. The low, but measurable, levels of plasma CPT-11 and SN-38 reached the C<sub>max</sub> of 277.6 $\pm$ 125.3 ng/ml and 1.6 $\pm$ 0.5 ng/ml (mean $\pm$ SD), respectively, at the lowest CPT-11 dose. The antiangiogenic effect of metronomic CPT-11 seems to be suggested by the TSP-1 plasma concentrations that were increased at the CPT-11 1.4 and 2.8 mg/m<sup>2</sup>/day schedules (e.g. at day 49, 153.4 $\pm$ 30.1% and 130.4 $\pm$ 9.2% vs. 100% of baseline values before treatment, respectively) and by the initial, but variable, increase in plasma VEGF (e.g. at day 21, 124.4 $\pm$ 41.7% and 132.3 $\pm$ 46.8%, respectively) probably due to the induced hypoxic conditions of tumour. Interestingly, the SN-38 plasma concentrations were statistically related to TSP-1 plasma levels in the 4 patients with stable disease (P=0.0062, r=0.3995). Metronomic CPT-11 schedules are feasible, very well tolerated and clinically considerable in 20% of CPT-11 resistant patients. Plasma SN-38 concentrations were measurable and related to the increase of the antiangiogenic factor TSP-1.

## References:

1. Man S, Bocci G, Francia G, et al: Antitumor effects in mice of low-dose (metronomic) cyclophosphamide administered continuously through the drinking water. *Cancer Res* 62:2731-5, 2002.
2. Bocci G, Nicolaou KC, Kerbel RS: Protracted low-dose effects on human endothelial cell proliferation and survival in vitro reveal a selective antiangiogenic window for various chemotherapeutic drugs. *Cancer Res* 62:6938-43, 2002.
3. Allegrini G, Goulette FA, Darnowski JW, et al: Thrombospondin-1 plus irinotecan: a novel antiangiogenic-chemotherapeutic combination that inhibits the growth of advanced human colon tumor xenografts in mice. *Cancer Chemother Pharmacol* 53:261-6, 2004.

**ALTERATION OF WNT/B CATENIN SIGNALLING AND EFFECT OF LITHIUM SALTS TREATMENT ON TgCRND8 MICE****Fiorentini Anna***Dipartimento di Farmacologia Preclinica e Clinica, Università degli Studi di Firenze, Viale Pieraccini 6, Firenze**E-mail: anna.fiorentini@unifi.it*

Senile plaques, made of fibrillary  $\beta$ -amyloid ( $A\beta$ ) peptide deposits, and neurofibrillary tangles, made of hyperphosphorylated tau protein are the histopathological hallmarks of Alzheimer's disease (AD). Many studies have shown that  $A\beta$  production and tau hyperphosphorylation in AD brain are promoted by GSK-3. This kinase was identified as a participant in the signal-transduction pathway regulated by ligands of the Wnt family. Dysfunction of one such pathway, the canonical Wnt/ $\beta$ -catenin signalling, leads to GSK-3 $\beta$  activation and to increased levels of tau phosphorylation in AD. Dickkopf-1 (DKK-1) protein negatively modulates the canonical Wnt pathway and might, therefore, be a component of the sequence of events leading to neuronal toxicity. Lithium salts, through inhibition of GSK-3 activity, reduce  $A\beta$  production and tau hyperphosphorylation. The aim of this study was to evaluate the alteration of Wnt pathway and to test the effectiveness of LiCl treatment in ameliorating the AD-like pathology in the TgCRND8 mice. To this aim, the expression pattern of DKK-1 and  $\beta$ -catenin was investigated. Western blotting and immunohistochemical techniques were used to reveal these proteins. The expression of DKK-1 was investigated in the brain of 7- and 12-months-old transgenic CRND8 mice, expressing the Swedish and the Indiana human mutant amyloid precursor protein (APP), and in 12-months-old transgenic APP<sup>swe</sup>/PS1-dE9 mice, expressing the Swedish human APP mutation and the human variant dE9 of presenil-1. Non-Tg wild type littermates mice were used as controls. A group of 2-months-old-TgCRND8 (n=8) and control mice (n=8) received i.p. injections of either 0.6 M LiCl (10  $\mu$ l per g of body weight) or sterile saline (10  $\mu$ l per g of body weight) daily for 30 days. A marked induction of DKK-1 immunoreactivity was found in the II, III, V and VI layers of parietal cortex, in the piriform cortex and in the CA1 area of the hippocampus of TgCRND8 mouse brain, as compared to controls. DKK-1 staining was localized in the cytoplasm and apical processes of neurons. Western blot analysis with DKK-1 antibody confirmed the immunohistochemical data. Double-labelling immunohistochemistry with DKK-1 and  $A\beta$ (1-42) antibodies revealed DKK-1 immunopositive neurons in the vicinity of the amyloid plaques. Numerous DKK-1-immunopositive neurons were detected in the CA3 area of the hippocampus and in the entorhinal cortex of APP<sup>swe</sup>/PS1-dE9 mice. These findings demonstrate that the Wnt signalling is impaired in the brain of aged transgenic mouse models of AD. In the TgCRND8 mice, lithium treatment increased  $\beta$ -catenin immunoreactivity in the dentate gyrus of the hippocampus and in the parietal cortex, as compared to saline-treated mice. These data show that lithium treatment restores the Wnt signalling in this transgenic mouse model of AD. The effect of lithium salts on DKK-1 levels is under investigation.

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**BINDING THERMODYNAMICS AT THE HUMAN A<sub>2B</sub> ADENOSINE RECEPTOR****Fogli Eleonora**

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The thermodynamic parameters  $\Delta G^\circ$ , equilibrium standard enthalpy ( $\Delta H^\circ$ ), and entropy of the binding equilibrium of 4 adenosine receptor agonists and five antagonists at adenosine A<sub>2B</sub> receptors transfected in HEK 293 cells (hHEK 293-A<sub>2B</sub> cells) were determined by means of affinity measurements at 6 different temperatures (4, 10, 15, 20, 25 and 30°C) and van't Hoff plot analysis.

Binding assays were performed in hHEK 293-A<sub>2B</sub> membranes at 4-30°C in a thermostatic bath assuring a temperature of  $\pm 0.1^\circ\text{C}$ . All buffer solutions were adjusted to maintain a constant pH of 7.4 at the desired temperature. Competition experiments of 3 nM [<sup>3</sup>H]MRE 2029F20 were performed in duplicate in a final volume of 100  $\mu\text{l}$  in test tubes containing 50 mM Tris HCl buffer, 10 mM MgCl<sub>2</sub>, 1 mM EDTA, 0.1 mM benzamidine pH 7.4, 100  $\mu\text{l}$  of membranes and at least 12-14 different concentrations of typical adenosine receptor agonists and antagonists. Non-specific binding was defined as the binding in the presence of 1  $\mu\text{M}$  ZM 241385 and was about 35% of total binding.

Van't Hoff plots were linear for agonists and antagonists in the temperature range 4-30°C. While  $K_D$  and  $K_i$  values changed with temperature,  $B_{\text{max}}$  values appeared to be largely independent suggesting a same population of receptors at all temperatures, with a range of 420-450 fmol/mg of protein at the temperatures investigated.  $\Delta G^\circ$  values ranged from  $-29.3 \pm 0.1$  to  $-41.3 \pm 0.2$  kJ mol<sup>-1</sup> for agonists and from  $-40.3 \pm 0.1$  to  $-47.2 \pm 0.2$  kJ mol<sup>-1</sup> for antagonists.  $\Delta H^\circ$  and  $\Delta S^\circ$  values showed that the binding of agonists was always totally entropy-driven while it was enthalpy and entropy-driven for antagonists,  $\Delta H^\circ$  values ranging from  $7.0 \pm 0.5$  to  $26.0 \pm 1.5$  and from  $-20 \pm 2$  to  $-40 \pm 4$  kJ mol<sup>-1</sup> and  $\Delta S^\circ$  values from  $123 \pm 7$  to  $225 \pm 20$  J kmol<sup>-1</sup> and from  $10 \pm 1$  to  $92 \pm 6$  J kmol<sup>-1</sup> for agonists and antagonists, respectively. Agonists and antagonists are therefore thermodynamically discriminated. This binding behaviour has previously been found to be typical of adenosine A<sub>1</sub>, A<sub>2A</sub>, and A<sub>3</sub> receptor antagonists. The results are discussed with the aim of obtaining new details on the nature of the forces driving the A<sub>2B</sub> binding at a molecular level.

This study provides the evidence that agonists and antagonists at the A<sub>2B</sub> receptors, like the other adenosine subtypes, are discriminated from a thermodynamic point of view.

## **BV8/PROKINETICIN SYSTEM AS A NEW MODULATOR OF THE IMMUNE RESPONSES**

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Bv8, a 77 aminoacid protein recently isolated from *Bombina variegata* skin, belongs to a novel family of secreted proteins whose homologues have been described in rodents and human. Two G-coupled receptor named PKR-1 and PKR-2 have been found for Bv8.

The biological activities linked to Bv8/Prokineticin have been rapidly increasing; in particular an hyperalgesic activity of Bv8 has been demonstrated. There is now evidence that Interleukin (IL-) 1 and Tumor necrosis factor alpha (TNF- $\alpha$ ) are involved in the creation of inflammatory pain while the anti-inflammatory cytokines limite this effect. Considering that lymphoid organs, circulating leukocytes and hematopoietic cells express high levels of Bv8-like proteins and of their receptors we consider immune system cells as a possible target for Bv8. We test the impact of Bv8 administration on several mouse macrophage and T cell functions. We investigate, through the use of knocked out mice, the receptor subtype involved in the observed effects and we try to explain the mechanism by which Bv8 acts.

BALB/C male mice, PKR-1 KO mice and their wild type controls were used in this study. The ability of Bv8 to induce macrophage chemotaxis was tested with a Boyden chamber, in some experiments after cells preincubation with PTX or phospholipase inhibitor U73122.

In the cytokines experiments, Bv8 at different concentration was added to macrophage cultures in presence of LPS or LPS + IFN $\gamma$  for IL-1 $\beta$ , TNF $\alpha$ , IL-10 and IL-12 evaluation or to spleen cells in presence of Con-A for IL-2, IFN $\gamma$  (24h incubation) and for IL-10 and IL-4 (48h incubation) evaluation. In in vivo experiments Bv8 was administrated s.c. at the dose of 250 pmoles/Kg for a period of 4h.

In the studies aimed at evaluating an antigen-specific answer, two weeks after mouse immunization with KLH spleen cells were incubated with or without KLH in presence/absence of Bv8  $10^{-11}$  M. In in vivo experiments Bv8 was administrated at the moment of the immunization or 4h before spleens collection.

After RT-PCR identification of PK2 and of its receptors on mouse peritoneal macrophages, we demonstrated a pro-inflammatory effect of Bv8 on these cells. In fact the protein was able to stimulate macrophage migration and to increase the production of the pro-inflammatory cytokines IL-1 and IL-12, decreasing that of the anti-inflammatory IL-10. Expermiments with cells from PKR-1 ko mice demonstrated the involvement of this receptor which is probably coupled to a Gq protein as suggested by our study in which the effects of Bv8 on chemotaxis and cytokines were not PTX (pertussis toxin)sensitive while they were completely prevented by the phospholipase C inhibitor U73122.

Bv8 was also able to impact Th1/Th2 balance, in fact, in both types of responses, we observed a significant decreament of the Th2 cytokines IL-4 and IL-10, while the Th1 cytokines IL-2 and IFN $\gamma$  were not modified. These effects were lost in PKR-1 ko mice. In conclusion we demonstrated that Bv8 is a potent modulatory factor of the immune responses.

## POST-TRANSCRIPTIONAL REGULATION OF BCL-2 RNA

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Most of the human follicular B-cell lymphomas is characterized by the chromosomal translocation t(14;18) that juxtaposes the bcl-2 gene with the IgH locus, creating a hybrid gene, with a consequent increase of bcl-2 cellular levels (1). Bcl-2 expression is regulated at a post-transcriptional level by Adenine-uridine Rich Element (ARE) motif, localized in the 3'UTR of the bcl2 RNA (2). The aim of this work was to understand the ARE-mediated decay, interfering with the normal decay of bcl-2 mRNA by target the ARE sequence with three chemically-modified oligoribonucleotides designed in the antisense orientation (asORNs).

Human follicular t(14;18)-positive B-cell lymphomas DOHH2, promyelocytic leukaemia HL60, neuroblastoma SHSY-5Y, and human embryonic kidney HEK293, were maintained in required medium. Three 2'-O-methyl antisense oligoribonucleotides have been designed complementary to the ARE core sequence of bcl-2 gene; a 26-mer degenerated ORN (degORN) was used as control. The transcription blocker DRB was purchased from Sigma Aldrich (St. Louis, MO).

396-bp U1 segment containing the 3-UTR ARE sequence was cloned in the pCRII to produce the pCR-U1 plasmid, the fragment was subsequently amplified and cloned in plasmid pCRII to produce plasmid pCR-U2. The 400-base pair fragment containing the 3'UTR ARE sequence was cloned at 3'-end of hRlucP-pGL4.71P to produce pGL4.71P b-ARE.

Cell-free RNA degradation assays were performed as described by Bevilacqua et al. (3). All cell lines were transfected by using Lipofectamine 2000 Reagent as previously described (4).

The three oligos reduced significantly bcl-2 degradation rate with a gene selective action. They all increased significantly the total amount of bcl2 mRNA doubling its half-life; they also acted on bcl-2 protein increasing its expression. Furthermore, they induced a significant increase of the reporter Rluc activity.

The experiments show that asORNs can increase bcl-2 expression inhibiting its degradation in a sequence specific manner and in a dose-response fashion.

### References

1. Capaccioli S, et al. (1996) *Oncogene* 13: 105-15.
2. Schiavone N, et al. (2000) *FASEB J* 14: 174-84.
3. Bevilacqua A, et al. (2003) *J Biol Chem* 278: 23451-9.
4. Bevilacqua A, et al. (2007) *Mol Pharmacol* 71: 531-8.

**AUTONOMIC REGULATION UNDER STRESS CONDITIONS IN HIGH ANXIETY MICE****Gaburro Stefano***Dipartimento di Farmacologia ed Anestesiologia, Università degli Studi di Padova, via L. Meneghetti 2, Padova**E mail: stefano.gaburro@unipd.it*

Anxiety is a complex combination of emotions that includes fear, apprehension and worry and somatic manifestations such as a rapid heart rate increase. Autonomic system (ANS) influences cardiovascular response under stress conditions through sympathetic and parasympathetic balance. One method to study this regulation of the ANS is by using heart rate variability (HRV) analysis. Patients suffering from anxiety a HRV reduction (1). The development of radio telemetry devices allows the evaluation of physiological parameters such as heart rate (HR), body temperature (BT), and HRV in freely moving rodents. Therefore, by using radio telemetry devices, two mice lines, selectively bred for high (HAB) or normal (NAB) anxiety-related behaviour, were monitored for cardiovascular responses under basal and challenge conditions. Thus, one aim of the present study was to evaluate different cardiovascular responses in HABs and NABs in the fear conditioning paradigm. We quantified possible markers (HR, BT, and HRV) for anxiety trait and also to evaluate possible gender differences in this mouse model. Eight female and male HABs and NABs (30-40 g) were implanted i.p. with radio-telemetry device (Datascience, San Diego USA) and allowed to recover for 1 week before commencement of experiments (2). The fear conditioning paradigm used consisted of 3 different phases: acquisition, extinction, and extinction recall. In the acquisition phase animals were placed in context A and exposed to five pairings of the conditioned stimulus (CS) (light, 2 min), which was co-terminated with a mild non painful unconditioned stimulus (US) (air-jet, 30 sec). Twenty four h later mice were placed in context B for the extinction fear memory phase. Mice were exposed to the CS for 15 presentations. Twenty four h following extinction, mice were placed in the same context as for extinction and exposed to 5 CS presentations. Behaviour was videotaped and fear index evaluated as the percentage time spent at freezing (absence of the body movements except for those associated with respiration). Physiological signals (BT and HR) were recorded via a receiver and digitalized at 2000 Hz frequency. HRV was evaluated using time domain analysis as RR intervals (interbeat interval) and SDRR (SD of interbeat interval).

In the acquisition of fear memory it was observed that both female HABs and NABs acquired fear memory. The analysis of telemetry data during acquisition showed that female HABs displayed an increased HR and reduced HRV (shorter RR intervals, SDRR) compared to female NABs. Conversely male HABs and NABs did not show any fear memory acquisition (no freezing behaviour observed) and did not show any cardiovascular difference. There was no difference in body temperature response for both female and male groups of mice. Twenty four h following the acquisition of fear memory, extinction trials were performed. At the first CS presentation, female NABs exhibited a lower percentage of freezing compared to the last presentation of acquisition. This result indicates that female NABs have less memory consolidation than female HABs that exhibits the same freezing level following the last CS-US pairing in acquisition. Furthermore, it was observed that female HABs did not extinguish their fear behaviour after 15 CS presentations while female NABs extinguish after 5 CS presentations. In terms of cardiovascular responses, female HABs showed lower HR and HRV compare to female NABs. There were no differences observed in body temperature among HABs and NABs. We also correlated fear and HR response that revealed a slight correlation. In extinction recall it was observed that female HABs displayed enhanced fear memory retention compared to NABs, which extinguished completely the fear memory. No differences in cardiovascular responses were observed. Collectively, these data showed a clear gender difference as female mice acquired fear behaviour whereas male mice acquired no fear behaviour using air-jet as an anxiety provoking stimulus. Cardiovascular responses in female HABs showed an altered heart rate response and reduced HRV compared to the female NABs demonstrating that HR with HRV could possibly be used as a marker for fear. Further analysis (frequency domain of HRV and hormonal evaluation) will be undertaken to aid in the understanding of the underlying mechanisms involved in the altered HR and cardiovascular responses in this mouse model.

## References:

1. Cohen H, Benjamin J (2006) *Autonom Neurosci* 128: 1-8.
2. Stiedl O, Tovote P, Ogren SO, Meyer M (2004) *Autonom Neurosci* 115: 15-27.



## MONITORING OF INFLAMMATION IN SHRSP BY MAGNETIC RESONANCE IMAGING

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The spontaneously hypertensive stroke-prone rat (SHR-SP), a model of brain and kidney disease, shows a progressive increase of 24-h proteinuria and accumulation in plasma and urine of acute-phase proteins (APP) when subjected to high-salt permissive diet (JPD). We previously demonstrated that there is a strong accumulation of macrophages in the kidney and in the brain of SHR-SP when they develop end-organ damage (1). Recently magnetic resonance imaging (MRI) for non-invasive detection of macrophages tissue infiltration was developed by labelling cells with an ultrasmall superparamagnetic iron oxide (USPIO).

The aim of this study was to use MRI to detect macrophage infiltration in the kidney and the brain after injection of USPIO in SHR-SP.

SHR-SP, fed a high-salt diet and receiving 1% NaCl in drinking water, once a week were weighed and housed individually in metabolic cages for 24 h to measure their food and liquid intake and to collect urine. With bovine albumin as a standard, 24-h urine protein was measured according to Bradford. Rats were randomized into one of three groups: group 1 (n=3) was injected intravenously with a single dose of USPIO (22.4 mg/kg) when the value of proteinuria was lower than 40 mg/day; group 2 (n=3) at 100 mg/day; group 3 (n=3) when brain damage was visualized with MRI. The signal intensity was measured in the kidney and in the brain before and 24 h after injection of the contrast agent. Gradient echo sequence was performed on a spectrometer operating at 4.7 T. MRI findings were compared with histological data.

MRI analysis revealed that twenty-four h after injection of USPIO, a significant decrease in signal intensity was observed in the cortex of kidneys of group 3 more than in group 2. There was no or low variation in the group 1. Only in group 1 the signal intensity in the brain changed in the same area where brain damage developed.

Prussian blue staining (Perl's staining) showed a major presence of iron plaques in the kidney of the group 3 compared with group 2, whereas in the group 1 the staining was almost undetectable. Immunohistochemical results indicated that some USPIO particles were taken by infiltrating macrophages, ED-1<sup>+</sup> cells. The double staining protocol for iron and ED-1<sup>+</sup> cells needs to be improved, because of the background signals and of the decreased detection of ED-1<sup>+</sup> cells.

In the brain, histological detection of USPIO was hampered by the fact that *in situ* detection of iron uptake by conventional Prussian blue staining was not sensitive enough to detect low amount of iron. We needed to enhance the signal of Prussian blue staining by diaminobenzidine (DAB), and thereafter the histological data obtained confirmed the presence of iron-containing cells in the ischemic brain lesions.

Our results suggest that MRI with intravenously administration of USPIO appears to be a valuable and promising tool that can be used as a non-invasive and sensitive method *in vivo* to detect macrophages in different inflammatory pathology, such as brain ischemia and kidney diseases.

### References:

1. Gianella A, et al. (2007) Am J Pathol 170: 1165-77.

## SEROTONIN TRANSPORTER 5HTTLPR POLYMORPHISM, CLINICAL VARIANTS AND SYMPTOM SEVERITY IN PATIENTS WITH IRRITABLE BOWEL SYNDROME

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Irritable bowel syndrome (IBS) affects approximately 15-20% of general population, causes abdominal pain, discomfort, altered bowel habits, and it is more common in women. Serotonin (5-HT) is a key mediator of intestinal peristalsis and after its secretion, it is effectively removed from the neuronal gap by serotonin transporter (SERT). Gene expression of SERT is modulated by a promoter polymorphism (44-base pair insertion/deletion; 5HTTLPR), which gives rise to long (L) and short (S) alleles. The S allele causes a decreased SERT expression with reduced efficiency of cellular serotonin re-uptake, a condition that seems to affect the response of psychiatric and peripheral disorders to drugs acting as SERT blockers. Since SERT inhibitors have been proposed for the treatment of IBS, this study was designed to evaluate possible associations of 5HTTLPR polymorphism with different clinical forms and symptom severity in IBS.

IBS patients were selected according to Rome II criteria, and subdivided into diarrhoea predominant (D-IBS), constipation predominant (C-IBS), and alternating bowel habit (A-IBS) groups. Symptom severity was estimated by the Francis-Whorwell score. Healthy volunteers matched for sex and age were also enrolled. Genomic DNA was extracted from whole blood or saliva, and the SERT gene promoter region containing the 5HTTLPR polymorphism was amplified by polymerase chain reaction (PCR) using primers designed on the basis of GeneBank nucleotide sequence. Expected sizes of PCR products were 528 bp and 572 bp for S and L alleles, respectively. DNA bands were visualized by electrophoresis with 2% agarose gel in the presence of ethidium bromide.

One hundred-fifty-two IBS patients (38 males, 114 females; mean age 40.2 years; age range 18-75 years) and 109 healthy volunteers (38 males, 71 females; mean age 44.8 years; age range 22-84 years) were genotyped. All subjects were Italians of Caucasian origin. Frequencies in IBS patients (L/L 32.2%, L/S 54.6%, S/S 13.2%) did not differ significantly from healthy volunteers (L/L 25.7%, L/S 54.1%, S/S 20.2%; Fisher's exact test:  $P=0.236$ ), with a slightly lower prevalence of S/S genotype in the former group. When stratifying patients by clinical variants, the genotype distribution was: D-IBS ( $n=59$ ), L/L 30.5%, L/S 50.8%, S/S 18.6%; C-IBS ( $n=57$ ), L/L 38.6%, L/S 54.4%, S/S 7.0%; A-IBS ( $n=36$ ), L/L 25%, L/S 61.1%, S/S 13.9%. Comparison of genotype frequencies in bowel habit subgroups vs. healthy volunteers indicated a significant difference for C-IBS ( $P=0.041$ ), but not for D-IBS or A-IBS patients. Mean symptom severity score values in IBS patients with L/L ( $256.4\pm 68.2$ ), L/S ( $275.1\pm 68.8$ ) and S/S ( $268.3\pm 71.2$ ) genotypes did not differ significantly (ANOVA:  $F=0.892$ ,  $P=0.412$ ).

Previous reports have provided conflicting evidence about a possible involvement of 5HTTLPR polymorphism in the pathogenesis and/or clinical presentation of IBS. The present results indicate a slightly reduced overall prevalence of S/S genotype in an Italian cohort of IBS patients, but suggest a significant association between the C-IBS clinical variant and 5HTTLPR polymorphism (low prevalence of S/S genotype in C-IBS patients vs. healthy volunteers). No relationship appears to exist between 5HTTLPR genotypes and symptom severity in IBS patients. This study allows hypothesizing that SERT polymorphism is not likely involved in the pathogenesis of IBS, suggesting the involvement of multifactorial determinants, but it may be a relevant factor affecting the predominance of specific clinical presentations (i.e. C-IBS, D-IBS or A-IBS). On these bases, it is conceivable that the use of SERT blockers could be effective in the management of IBS, in particular in the subgroup of C-IBS patients.

## IRON OXIDE LOADING AND MAGNETIC RESONANCE IMAGING FOR ENDOTHELIAL PROGENITOR CELLS DETECTION IN A MOUSE MODEL OF HIND LIMB ISCHEMIA

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Recent studies suggest that most stem cells injected *in vivo*, into the heart or the hind limb, survive only for a few days. However, no systematic study has been performed to quantify stem cell death *in vivo* and the time course of this phenomenon. Ideally this objective should be achieved with a non-invasive technique that provides for repeated observations and can track few cells with a high spatial resolution.

Therefore, the aim of the present study was to establish whether human endothelial progenitor cells from the peripheral blood (hEPCs) loaded *in vitro* with superparamagnetic iron oxide (SPIO-Feridex) can be visualized *in vivo*, in a mouse model of hind limb ischemia, by magnetic resonance imaging (MRI).

hEPCs were incubated in M199 medium containing 25 µg Fe/ml of SPIO-Feridex and 375 ng/ml poly-L-lysine. After 48 h incubation hEPCs ( $1 \times 10^5$ - $9 \times 10^5$ ) were washed, harvested and put in 100 µl agarose gel to determine the minimum number of labelled cells necessary to obtain a detectable change in MRI signal intensity. MRI showed significant T2 signal intensity loss from  $1 \times 10^5$  iron labelled cells compared to non-labelled cells; signal intensity loss was proportional to SPIO-Feridex-loaded cell number. SPIO-Feridex-loaded hEPCs maintained a normal phenotype as established by Ac-LDL-DiI uptake, UEA-1 lectin staining, and CD31 expression. *In vivo* studies were performed in a mouse model of acute hind limb ischemia induced by left femoral artery excision in CD1 mice. To prevent hEPCs rejection, mice were daily immunosuppressed with cyclosporin A (20 mg/kg). At the time of surgery, mice were injected in three different points in the adductor muscle with a total of 15 µl PBS containing  $6 \times 10^5$  SPIO-Feridex-labelled (n=3) and non-labelled (n=5) EPCs. MRI was performed every other day for 7 to 11 days until sacrifice. MRI hypointense signal was present up to 11 days after cells injection. To verify the correspondence between hypointense regions and the presence of iron nanoparticles, Pearl's Prussian blue staining was performed on histological sections of adductor muscles injected with SPIO-Feridex-loaded hEPCs. The results showed a positive correlation between iron staining and MRI signal.

These preliminary results suggest that MRI of paramagnetic beads-loaded hEPCs is a feasible strategy to follow the engraftment potential of hEPCs into ischemic tissues.

## ROLE OF THE ENDOCANNABINOID/VANILLOID SYSTEM ON LIMBIC CORTEX APOPTOSIS IN NEUROPATHIC MICE

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Aim of this research was to study the potential involvement of vanilloid and endocannabinoid systems in alleviating neuropathic pain in mice.

A mouse model of neuropathic pain consisting of spared nerve injury (SNI) of the sciatic nerve was used to examine the anti-allodynic and anti-hyperalgesic effect of a chronic treatment with N-arachidonoyl-serotonin (AA5HT), an inhibitor of the fatty acid amide hydrolase, and a TRPV1 receptor blocker.

Neuropathic mice treated chronically with vehicle developed thermal hyperalgesia and mechanical allodynia at 3, 7, 14, and 21 days post-surgery.

RT-PCR analysis showed increased expression of the bax/bcl-2 ratio ( $40\pm 2\%$ ), caspase-1 ( $84\pm 3\%$ ), caspase-8 ( $53\pm 6\%$ ), caspase-9 ( $25\pm 6\%$ ), caspase-12 ( $58\pm 2\%$ ), and TNF-R1 ( $32\pm 2\%$ ) genes in the cortex by 7 days post-injury.

SNI induced an increased expression of the bax/bcl-2 ratio ( $30\pm 2\%$ ), caspase-12 ( $75\pm 2\%$ ), and TNF-R1 ( $39\pm 2\%$ ) genes in the cortex also by 14 days post-injury, without affecting the mRNA levels of the regulative caspases, such as caspase-1, caspase-8, and caspase-9.

Western blot analysis showed increased caspase-3 protein levels in the cortex at 3, 7, and 14 days post-surgery.

The fatty acid amide hydrolase inhibitor and AA5HT (5 mg/kg i.p. once daily) prevented the development of thermal hyperalgesia and mechanical allodynia at 7, 14, and 21 days post-surgery, and reduced caspase-3 protein levels.

AA5HT treatment reduced the mRNA levels of the bax/bcl-2 ratio, caspase-1, caspase-8, caspase-9, and caspase-12.

These results suggest that the dual activity of AA5HT, on endocannabinoid metabolism inhibitor and TRPV1 receptors antagonist, could be a novel therapeutic strategy for newer pain-relievers.

## **EFFECTS OF GLYCYRRHIZIN AND 18 $\beta$ -GLYCYRRHETINIC ACID ON UVB-IRRADIATED HUMAN KERATINOCYTES**

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UV radiation is directly related to the development of skin cancer and the carcinogenic effect is primarily attributed to the UVB portion of the solar radiation. It is known that liquorice root is rich in compounds which exert several pharmacological actions. The aim of our study was to investigate the effect of glycyrrhizin (the main constituent of liquorice root) and of its metabolite aglycone, 18 $\beta$ -glycyrrhetic acid, on UVB-irradiated human keratinocytes.

For this purpose we examined in cultured epidermal cells the effects of UVB doses at 50 mJ/cm<sup>2</sup> and 75 mJ/cm<sup>2</sup>. MTT test and [<sup>3</sup>H]-thymidine incorporation were performed as a test of the proliferation rate, whereas DCF assay was employed to determine formation of intracellular reactive oxygen species. Moreover we investigated the possible anti-apoptotic effect of these compounds after UVB-exposure by Western-blot analysis.

Our results showed that glycyrrhizin and 18 $\beta$ -glycyrrhetic acid are not toxic for keratinocytes cells at concentration of 30  $\mu$ M. DCF assay showed that 18 $\beta$ -glycyrrhetic acid but not glycyrrhizin was a potent anti-oxidant agent. In human keratinocytes bcl-2 expression was down-regulated by 18 $\beta$ -glycyrrhetic acid, but not by glycyrrhizin. These results showed that 18 $\beta$ -glycyrrhetic acid is a potential agent that can inhibit UVB-induced cutaneous oxidative stress and tumor promotion. Further studies must be performed to understand the mechanism of the protective effect.

## A PRECISION-CUT SMALL INTESTINE SLICE METHOD FOR STUDING XENOBIOTIC METABOLISM

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Several *in vitro* systems are available for studying the metabolism or toxicity of novel compounds in the small intestine. Although subcellular fractions, such as microsomes have been most extensively used, precision-cut small intestine slices may be useful tool possessing the following advantages: no requirement of proteolytic enzymes for diving preparation; normal polarity of the cells remain intact; intact cell heterogeneity and cell-cell interactions (1). The use of intestine slices is not new but it has become more widespread after the introduction of apparatus that minimally traumatized the tissue and produced slices very uniform for shape and dimension (2). The aim of the present study is to set up the preparation of intestine slices in order to study xenobiotic metabolism.

To prepare agarose-filled slices, ileum was cut in parts subsequently ligated on one side. These parts were then filled with 3% (w/v) low melting agarose solution in 0.9% (w/v) NaCl at 37°C and were allowed to gel in ice-cold Krebs-Henseleit buffer. The agarose-filled intestines were cut in 1 cm parts put in a 24 multiwell plate that was filled with agarose. The cylinders obtained were used to prepare precision-cut slices, using a Krumdieck tissue slicer filled with oxygenated, ice-cold Krebs-Henseleit buffer (3).

After a pre-incubation period of 30 min, slices were individually incubated in RPMI1640 complete medium under carbogen atmosphere and incubations were carried out at 37°C in 12 wells plates with a continuous gentle shaking (4).

After various time (0.5, 1, 2, 4, and 24 h) medium samples were taken and slices homogenized. Total lactate dehydrogenase (LDH) and alkaline phosphatase (AP) leakage were measured both in pre-incubation and incubation medium and in whole slice homogenates.

In another series of experiments, intestinal slices were incubated in the presence of 7-ethoxycoumarin in order to measure the metabolism capacity.

The measure indicated that the slices were 400-450  $\mu\text{m}$  thick. The LDH leakage increased with time up to 6 h of incubation (60% of the total) and AP leakage increased with time up to 24 h. In conclusion the results of this study show that precision-cut small intestine slices prepared in our laboratory remain viable and metabolic competent up 24h.

### References:

1. De Kanter R, Monshouwer M, Meijer DK, Groothuis GM (2002) *Curr Drug Metab* 3: 39-59.
2. De Kanter R, et al. (2004) *J Pharmacol Methods* 51: 65-72.
3. Lerche-Lagrand C, Toutain HJ (2000) *Toxicology* 153: 221-53.
4. De Kanter R, et al. (1999) *Toxicology In Vitro* 13: 737-44.

**PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR GAMMA COACTIVATOR 1ALFA (PGC1ALFA) IN A MODEL OF DIET-INDUCED INSULIN RESISTANCE IN THE RAT**

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Metabolic pathways are controlled at different levels in response to environmental or hormonal stimuli. This control is achieved, at least in part, at the transcriptional level of gene expression. In mammals one of the most characterized examples of regulation of metabolic pathways by transcriptional coactivators (proteins that interact with transcription factors and regulate transcription) is peroxisome proliferator activated receptor gamma (PGC1alfa). PGC1alfa is activated by signals that control energy and nutrient homeostasis. PGC1 controls important metabolic pathways in several tissues; it has been shown to regulate adaptive thermogenesis in brown fat, hepatic gluconeogenesis and ketogenesis, and differentiation of slow-twitch muscle fibers. The fact that PGC1alfa controls important pathways in several tissues suggests that it can be a therapeutic target for anti-obesity or anti-diabetes drugs. It has been well established that PGC1alfa is strongly induced by cold in brown tissues, so to validate a model that can be useful to test new antidiabetic drugs, we first measured the level of PGC1alfa mRNA in brown fat by RT-PCR in rats after overnight cold exposure (4°C). RT-PCR is the most sensitive technique for mRNA detection and quantisation. After cold exposure PGC1alfa mRNA of brown fat was acutely induced ( $P < 0.001$ ) in cold exposed animals vs. control animals.

A high fat diet (HF) is suggested to increase the risk of obesity and diabetes mellitus. We analysed the effects of a long-term consumption of HF diet in rats as a possible model of insulin resistance. We measured the mRNA levels of PGC1alfa in brown fat, liver, and muscle of rats fed for 8 weeks with HF or with standard (STD) diet. Rats exposed to HF diet showed an increased body weight compared to rats exposed to STD diet. Moreover rats exposed to HF diet had higher blood glucose levels 2 and 3 h after an oral glucose load compared to STD diet rats. Our results show that PGC1alfa mRNA levels were induced in brown fat after HF diet. A more detailed knowledge about the function and regulation of PGC1alfa could provide new opportunities to manipulate energy expenditure and treat disorders of energy homeostasis such as obesity and obesity-linked diabetes.

**SINTHESY AND EXPRESSION OF A RECOMBINANT SONIC HEDGEHOG IN E. COLI****Grigoletto Jessica***Department of Pharmacology and Anesthesiology, University of Padua, Largo Meneghetti 2, 35121 Padua, Italy**E-mail: grigoletto.jessica@unipd.it*

Proteins of the Hedgehog (Hs) family are important signalling molecules during embryonic development of animals (1). Sonic Hedgehog (Shh), one of three Hh's, is the most intensively studied genes in the development biology, where Shh functions as a morphogen. This protein plays a central role in development of the nervous and skeletal system, where patterns the brain, the spinal cord, craniofacial elements, the axial skeleton, limbs and digits (2). Shh, expressed in the embryonic notochord, induces floor plate formation at the ventral midline of the neural tube and promotes the subsequent differentiation of ventral neurons in a region-specific manner, e.g. dopaminergic neurons in the midbrain and motor neurons in the spinal cord (3). Furthermore the Shh signalling pathway is involved in regulating granule cell precursor (GCP) proliferation in the cerebellum by Purkinje cell, where Shh is produced (4). The aim of the study was to elucidate the possible role played by Shh signalling pathway in cell proliferation process in neural progenitor cells isolated from rat postnatal cerebellum. Natural Shh is synthesized as a long inactive 45 kDa precursor protein and converts itself into an active molecule, through autocatalytic internal cleavage, to yield a 20 kDa N-terminal fragment (Shh-N) that is responsible for all its biological activity. Shh-N remains membrane associated through the addition of two lipids, a palmitic acid at its N terminus and a cholesterol at its C terminus (3).

In order to evaluate this activity, here we have cloned rat Shh by RT-PCR using rat mRNA as template in a procariotic expression vector under the control of the T7 promoter. The mature domain of rat Shh (23-188) has been mutagenized at its N terminus by adding two additional amino acid (Ile Ile) in the place of Cys<sup>23</sup> in order to mimic the presence of palmitic acid, while at C terminus was added six His residues. Rat Shh was synthesized at low temperature (25°C) and purified to homogeneity in non-denaturising conditions using the His tail and a Ni<sup>2+</sup>-NTA column. The two unique Cys are free and molecular mass determined by MALDI mass spectrometry was 20951.5 Da in agreement with theoretical mass 20950.1 Da. Circular dichroism shows that rat Shh is correctly folded with high presence of  $\alpha$ -helical structure. Preliminary data shows that our recombinant Ile<sup>23</sup>-His<sup>188</sup>(6) is biological active on neural progenitor cells isolated from postnatal cerebellum. Now we started to examined the role of Sonic Hedgehog to control the growth of granule cell precursors (GCP) in the cerebellum.

## References:

1. SaeKi K, et al. (2000) *Biochim Biophys Acta* 1476: 219-29.
2. Dorus S, et al. (2006) *Hum Mol Genet* 15: 2031-7.
3. Williams KP, et al. (1999) *J Cell Science* 112: 4405-14.
4. Corrales JD, Rocco GL, Blass S, Guo Q, Joyner AL (2004) *Development* 131: 5581-90.



**THE PROKINETICIN RECEPTOR AGONIST BV<sub>8</sub> INCREASES GABA RELEASE IN THE PERIAQUEDUCTAL GREY AND MODIFIES RVM CELL ACTIVITIES AND THERMOCEPTIVE REFLEXES IN THE RAT**

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In this study the effect of prokineticin Bv8, a small protein secreted by skin of the *Bombina variegata* frog, has been investigated.

Bv8 is a potent agonist to both the identified receptors, the G-protein-coupled PK-R1 and PK-R2. We studied the effect of Bv8 on nociceptive responses and the ongoing or tail flick-related changes of rostral ventromedial medulla (RVM) ON and OFF cells activities in rats.

We found that intraperiaqueductal grey (PAG) Bv8 (100 e 200 pmol/rat) exerted a potent dose-dependent pro-nociceptive action and caused opposite effect on the ongoing RVM ON and OFF cell activities.

Bv8 increased the tail flick-induced ON cell burst of activity and increased the pause of the OFF cell. These effects were prevented by co-injecting Bv8 with Ala 24 (100 pmol/rat), a PK-R1/2 antagonist.

Microdialysis analysis applied in combination with the plantar test, showed that intra-PAG perfusion with Bv8 (0.25 and 0.5 pM) increased GABA, but not glutamate, extracellular levels, and also it decreased thermoceptive threshold. These effects were prevented if Bv8 was co-perfused with Ala24.

In conclusion, these data show that the stimulation of PAG PK-Rs might worsen pain perception and this effect is consistent with specific changes on the ON and OFF cell ongoing and tail flick-related activities, as well as with the Bv8-induced increase in PAG GABA levels.

**ROLE OF CB1 RECEPTOR SYSTEM IN A PHARMACOLOGICAL MODEL OF SCHIZOPHRENIA****Guidali Cinzia***DBSF, University of Insubria, Via A. da Giussano 10, 21052 Busto Arsizio (VA)**E-mail: cinzia.guidali@uninsubria.it*

Clinical and laboratory findings suggest that cannabinoid signalling is implicated in schizophrenia; however the interaction remains poorly understood, as data are often contradictory. On the basis of the intriguing but still confusing results present in literature, the aim of the present study was to investigate the role of the CB1 receptor system and signalling in a pharmacological model of schizophrenia based on repeated injections of the non-competitive NMDA antagonist, phencyclidine (PCP, 2.5 mg/kg chronic intermittent treatment for 1 month). This is a validated model that reproduces the metabolic hypofunction within the prefrontal cortex and it has been linked with the cognitive deficit and negative symptoms that are observed in schizophrenic patients (1). Seventy-two h after the last PCP injection the animals were exposed to object recognition test, a popular protocol to study recognition memory, which is a parameter usually altered in schizophrenia. PCP treated animals showed an impairment in the object recognition test as demonstrated by the reduction (-60%) in the difference score (difference between novel object and familiar object interaction) and in the recognition index (difference between the time exploring the novel and the familiar object corrected for total time exploring both objects). On the brains of the same animals we performed the CB1 receptor binding and the CP-55,940-stimulated [<sup>35</sup>S]GTPγS binding in order to study cannabinoid receptor functionality. Our results show that CB1 receptor levels were not modified by the pharmacological treatment but significant alterations in the coupling to G proteins were found in specific cerebral areas such as prefrontal cortex (-23%), globus pallidus (+71%), hippocampus (-34%), substantia nigra (-28%), and cerebellum (-40%), i.e. brain areas involved in the control of motor, emotional and cognitive states. Subsequently, to mimic the cannabis use observed in schizophrenic patients, we co-treated the PCP animals with a low dose of THC (0.5 mg/kg daily for three weeks) that *per se* was not able to induce any significant behavioural alteration. Co-treated animals showed a worsening in cognitive parameters evaluated by object recognition (-80%) accompanied by a sustained reduction (-50%) in CB1 receptor efficiency in the prefrontal cortex respect to PCP animals (23%). Moreover, we also tested the acute effect of THC (0.5 mg/kg) on the positive psychotic symptoms induced by a single PCP administration (3.5 mg/kg). THC injection *per se* did not affect the locomotor behaviour but significantly counteracted the hyperlocomotion and stereotypy induced by PCP. Our findings suggest that altered cannabinoid signalling is present in chronic PCP model of schizophrenia in selected cerebral regions involved in the modulation of cognitive and emotional states. In addition, the prolonged cannabis use worsen the cognitive functions altered in schizophrenics whereas a single THC injection could improve positive schizophrenic-like symptoms. In conclusion, this study confirms the presence of a complex relationship between cannabinoid systems and schizophrenia.

## References:

1. Cochran SM, et al. (2003) *Neuropsychopharmacology* 28: 265-75.

**MIGRATION AND INVASIVENESS OF HUMAN SMOOTH MUSCLE TSC2<sup>-/-</sup> CELLS IN NUDE MICE: EFFECT OF ANTI-EGFR AND RAPAMYCIN****Isaia Eleonora***Dip. di Medicina, Chirurgia e Odontoiatria, Facoltà di Medicina, Università degli Studi di Milano, Via di Rudini 8, 20142, Milano**E-mail: eleonora.isaia@unimi.it*

Tuberous sclerosis complex (TSC) is an autosomal-dominant tumour disorder characterized by multiorgan development of tumours affecting the kidneys, brain, heart and lungs. Mutations in the *TSC1* or *TSC2* gene, encoding hamartin and tuberin respectively, lead to increased mammalian target of rapamycin (mTOR) kinase activity, which regulates cell growth and protein translation. TSC is related to lymphangiomyomatosis (LAM), a rare disease characterized by widespread pulmonary proliferation of abnormal smooth muscle cells leading to cystic destruction of the lung parenchyma. In LAM patients, somatic mutations in *TSC1* or, more frequently, *TSC2* have been identified in abnormal lung tissue and renal angiomyolipomas (AML). Human TSC2<sup>-/-</sup> cells can be a source of useful information for developing more appropriate pharmacological strategies aimed at blocking the life-threatening growth of smooth muscle cells in TSC and LAM. In our laboratory, human TSC2<sup>-/-</sup> smooth muscle cells from a renal AML have been isolated. These cells are characterized by EGF-dependent proliferation, hyperphosphorylation of S6 kinase and its substrate S6, and positivity to HMB45 antibody, a marker of TSC and LAM cells. In these cells, antibodies directed against EGF- and IGF-1 receptors (anti-EGFR and anti-IGF-1R) cause the progressive cell death. To evaluate such a therapeutic potential and TSC2<sup>-/-</sup> cell invasiveness, we developed an *in vivo* model of TSC and LAM complications by applying TSC2<sup>-/-</sup> cells to nude mice. Five weeks old nude mice were injected i.p. with 5x10<sup>5</sup> TSC2<sup>-/-</sup> cells previously labelled with red dye PKH26-GL. Cervical, mediastinic and retroperitoneal lymph nodes and lungs, kidneys, and uteri were dissected out and properly processed after 30, 90 and 180 days from injection. Labelled TSC2<sup>-/-</sup> cells were detected. A widespread positivity to HMB45 antibody and a high S6 phosphorylation were observed in lymph nodes and uteri and, at a lesser extent, in lungs that showed an emphysema-like picture with cystic destruction of the parenchyma. Moreover, 2x10<sup>5</sup> TSC2<sup>-/-</sup> cells were nasally administered and in such a way the respiratory system was quickly invaded. TSC2<sup>-/-</sup> cells massively infiltrated into pulmonary alveolar walls and mediastinic lymph nodes, associated with a highly diffuse S6 phosphorylation and HMB45 positivity. Thirty and 60 days after nasal administration, a strong progressive destruction of lung parenchyma, likely associated with the high TSC2<sup>-/-</sup> cell proliferation, was observed. Interestingly, using the lymphatic endothelial marker LYVE-1, an increase of lymphatic vessels and an infiltration of lymphatic endothelial cells in the pulmonary parenchyma were found near the area where TSC2<sup>-/-</sup> cells were detected. Following both i.p. and nasal cell administration, anti-EGFR treatment completely abolished S6 phosphorylation and reversed the HMB45 positivity both in lungs and, after, in lymph nodes. Moreover, anti-EGFR caused the inhibition of lymphangiogenesis and a dramatic reduction in the number of TSC2<sup>-/-</sup> cells in lung. Rapamycin, the specific mTOR kinase inhibitor, had poorly effect, although it has been shown to normalize deregulated mTOR signalling in cells lacking tuberin. However, rapamycin was effective in reducing the positivity to LYVE-1 in lungs, according to its immunosuppressant action. In conclusion, human TSC2<sup>-/-</sup> smooth muscle cells exhibit an *in vivo* migratory and proliferative capability and invasiveness; as shown *in vitro*, anti-EGFR abolishes the proliferative and survival TSC2<sup>-/-</sup> cell properties *in vivo*, suggesting a new therapeutic approach to control the abnormal smooth muscle cell growth in TSC and LAM.

## PHARMACOLOGICAL PROFILE OF 3,5-DI-*t*-BUTYLCATECHOL (DTCAT) IN THE MAMMALIAN SKELETAL MUSCLE

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Malignant hyperthermia (MH) is an autosomally dominant hereditary disease triggered by volatile anaesthetics. MH may present as an acute and dramatic clinical event followed by death or as an abortive form with only few clinical signs (1). Mutated ryanodine receptor  $\text{Ca}^{2+}$  channels (RyRC) have been identified as molecular targets responsible for this disease. The diagnosis of MH is based on the *in vitro* contracture test (IVCT) of biopsied muscle bundles of the *vastus lateralis* muscle. The RyRC agonists employed so far (i.e. caffeine and halothane) in the standardised protocol, however, can only diagnose a patient as susceptible (MHS) or normal (MHN) to MH without solving the problem represented by the equivocal patient (MHEH), that is neither MHS nor MHN.

In a previous study, 3,5-di-*t*-butylcatechol (DTCAT) has been shown to stimulate  $\text{Ca}^{2+}$  release from rat skeletal muscle sarcoplasmic reticulum (SR) vesicles by binding to the RyRC (2). This property of DTCAT has suggested its use as an alternative tool in the IVCT to improve the diagnosis of MH. Then, the effects of DTCAT were investigated by recording: 1) the contractile response to electrical stimulation of rat diaphragm strips and phrenic nerve-diaphragm preparation [baseline and muscle tension (RelAfit) were recorded and measured with MacLab/Chart Vs 4.2]; 2) IVCT, performed according to the European MH group protocol; 3) intracellular  $\text{Ca}^{2+}$  concentrations ( $[\text{Ca}^{2+}]_i$ ) of primary human skeletal muscle cells, isolated from biopsied samples used for the IVCT, loaded with Fura2/AM (fluorescence signals were detected with an imaging system).

In rat diaphragm strips, DTCAT increased baseline tension and decreased RelAfit, in a concentration-dependent manner. This effect was similar, although less potent than that observed in tissues following treatment with caffeine, 4-chloro-*m*-cresol, and ryanodine. In the phrenic nerve-diaphragm preparation, when muscle was stimulated indirectly, DTCAT decreased, in a concentration-dependent manner, RelAfit; a similar effect was obtained when muscle was stimulated directly. In the presence of *d*-tubocurarine, DTCAT increased, in a concentration-dependent manner, the baseline tension without affecting RelAfit. 2,6-Di-*t*-butyl-4-methoxyphenol, which stimulates the SR  $\text{Ca}^{2+}$ -ATPase (3), was able to antagonise the effects of DTCAT. On the contrary, ruthenium red, which blocks the RyRC (4), was ineffective. In the IVCT, DTCAT increased the baseline tension of muscles from MHN, MHS, and MHEH patients with a similar potency [ $\text{pEC}_{50}$  (M)  $3.82 \pm 0.09$  MHN,  $4.03 \pm 0.08$  MHS, and  $3.84 \pm 0.08$  MHEH, respectively]. When determining  $[\text{Ca}^{2+}]_i$ , in the presence of extracellular  $\text{Ca}^{2+}$ , a maximal effect was already achieved at intermediate DTCAT concentrations, while in the absence of extracellular  $\text{Ca}^{2+}$ , the highest increase of  $[\text{Ca}^{2+}]_i$  was obtained at 300  $\mu\text{M}$  DTCAT either in MHS or MHN cells. Re-addition of extracellular  $\text{Ca}^{2+}$  to the cells, following DTCAT removal, led to further increase of  $[\text{Ca}^{2+}]_i$ . The latter effect was prevented by the application of  $\text{La}^{3+}$  and  $\text{Cd}^{2+}$ .

The present functional data confirm that DTCAT promotes  $\text{Ca}^{2+}$  release from SR via RyRC in intact mammalian skeletal muscles; however, it cannot represent an alternative tool to improve the diagnosis of MH.

### References:

1. Anetseder M, Ritter L, Horbaschek H, Hartung E, Roewer N (2000) *Acta Anaesthesiol Scand* 44: 338-42.
2. Fusi F, Iozzi D, Sgaragli G, Frosini M (2005) *Biochem Pharmacol* 69: 485-91.
3. Fusi F, Tzankova V, Valoti M, Pessina F, Sgaragli G (2001) *Biochem Pharmacol* 62: 1613-9.
4. Ma J (1993) *J Gen Physiol* 102: 1031-56.

**THERAPEUTIC MONITORING OF CLOZAPINE, ABCB1 HAPLOTYPE AND RESPONSE TO TREATMENT IN PSYCHOTIC PATIENTS****Lastella Marianna***Dipartimento di Medicina Interna, Università degli Studi di Pisa, Via Roma 55, 56100 Pisa  
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Clozapine, an atypical antipsychotic drug, is used to treat psychotic patients who are resistant to typical antipsychotics. The drug is substrate of the transmembrane transporter ABCB1, also known as P-gp, whose activity is related to the presence of single nucleotide polymorphisms (SNPs), namely C3435T on exon 26, G2677T on exon 21 and C1236T on exon 12. The aim of the present study was to evaluate possible correlations among plasma levels of clozapine and its active metabolite norclozapine, ABCB1 haplotype and polymorphisms, and response to the drug in patients affected by schizophrenia, schizoaffective disorders and bipolar disorders with psychotic features. Forty-one consecutive patients, 27 men and 14 women (age  $34.5 \pm 8.0$  and  $49.1 \pm 14.7$  years, respectively), were enrolled. A blood sample was withdrawn when clozapine achieved its steady state plasma levels, at least 14 days after the start of drug administration. Clozapine and norclozapine plasma concentrations were determined by a validated UV-HPLC method, while C3435T, G2677T, and C1236T SNPs were determined by RFLP-PCR or using RT-PCR genotypization assay on a Taqman platform (Applied Biosystems). Treatment response was evaluated by using CGI scale. Results obtained from statistical analysis were expressed as mean  $\pm$  standard deviation values, and P values lower than 0.05 were considered significant. Plasma levels of clozapine and norclozapine were  $197.6 \pm 143.2$  and  $107.1 \pm 84.4$  ng/ml, respectively. In this study, 6 patients (4 males and 2 females) experienced a negligible benefit from treatment ("minimally improved" or "no change" according to the CGI score), despite they received higher doses of the drug ( $196 \pm 135$  mg/die) with respect to the responders ("much improved" or "very much improved" according to the CGI score) ( $160 \pm 104$  mg/die). Noteworthy, dose-normalized plasma levels of clozapine and norclozapine were significantly higher in responder patients ( $1.71 \pm 1.4$  and  $0.75 \pm 0.46$  ng/ml/mg, respectively) than in 6 nonresponders ( $0.83 \pm 0.53$  and  $0.40 \pm 0.21$  ng/ml/mg, respectively), suggesting an alteration of absorption or excretion of the drug and catabolite. However, haplotypes of ABCB1, as well as the three single SNPs, failed to predict the response to clozapine in the present patients. These results demonstrate that dose-normalized plasma levels of clozapine and its metabolite predict treatment effectiveness, while further studies are warranted to investigate the role of ABCB1.

**THE ISOTHIOCYANATE SULFORAPHANE AFFECTS APOPTOSIS INDUCTION OF EX-VIVO LEUKAEMIA SAMPLES****Lenzi Monia**

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The isothiocyanate sulforaphane exerts cytostatic and cytotoxic effects on different tumour cell lines (1). For example, in our previous studies it has been shown to function as a potent antileukaemic agent *in vitro* (2). However, the behaviour of sulforaphane on primary leukaemia cells was never investigated. Cells from patients affected by leukaemia possess very different characteristics if compared to permanent cell lines. For example, it has been demonstrated that only cells featuring chromosomal translocations can be established as permanent cell lines (3). Primary cells can differently respond to sulforaphane. We therefore studied apoptosis induction by sulforaphane on primary leukaemia cultures coming from 15 patients suffering from different types of leukaemia.

Human peripheral blood was obtained from patients affected by different types of leukaemia: 2 cases of chronic lymphatic leukaemia (LLC), 7 cases of acute myeloid leukaemia (LMA), 2 cases of acute lymphoblastic T-cell leukaemia, 1 case of acute lymphoblastic B-cell leukaemia, 1 case of acute biphenotypic leukaemia, 1 case of aggressive lymphoma nasal type with strong leukaemisation. Human mononuclear cells were isolated by density gradient centrifugation using Ficoll-Hypaque. Human lymphocytes were cultured in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 1% antibiotics, and 1% glutamine. Cells were treated with different concentrations of sulforaphane (3-10-30  $\mu$ M) and the effects of sulforaphane on apoptosis induction were analyzed by flow cytometry.

We evaluated the proapoptotic effect of sulforaphane and we demonstrated that it does not exert any activity on samples coming from patients suffering from LLC. In fact, the fraction of apoptotic cells after 48 h of treatment with sulforaphane 30  $\mu$ M was only slightly increased with respect to the control (42.3% vs. 32.4%). On the contrary, sulforaphane was particularly active on samples coming from patients suffering from LMA, where the effect was also surprisingly marked on pharmacoresistant patients. For example, in one case the fraction of apoptotic cells after 48 h of treatment with sulforaphane 30  $\mu$ M was five-fold increased with respect to the control.

Moreover, the activity of sulforaphane on samples coming from patients suffering from acute lymphatic leukaemia was dependent on the cells involved. The most significant effect was registered on B-cell leukaemic, where the fraction of apoptotic cells increased from 27% in untreated cells to 55% in treated cells. The activity of sulforaphane on T-cell leukaemic was definitely weaker.

Finally, sulforaphane did not exert any activity on samples coming from patients suffering from nasal type lymphoma.

Taken together, our *ex-vivo* finding indicates that sulforaphane does possess interesting antileukaemic potential that should encourage further investigations and its introduction on clinical pilot study.

## References:

1. Gamet-Payrastré L (2006) *Curr Cancer Drug Targets* 6: 135-45.
2. Fimognari C, et al. (2002) *Carcinogenesis* 23: 581-6.
3. O'Connor R, et al. (1991) *Blood* 77: 1534-45.

**ACUTE BUT NOT CHRONIC TREATMENT WITH OLANZAPINE  
REDUCES EXPERIMENTAL ANXIETY IN RATS: POSSIBLE  
MECHANISMS**

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Olanzapine is an atypical antipsychotic whose particular action is due to the blockade of both dopaminergic (namely D2/D4 type) and serotonergic receptors (namely 5HT2 type). Previous studies from our laboratory demonstrated that the pharmacological effects of olanzapine could involve the glutamatergic system. Furthermore, olanzapine administration increases plasma and brain concentration of progesterone and of its metabolite allopregnanolone. It is notable that clinical studies have recently reported that olanzapine is effective in treatment-resistant depression (TRD) and in panic disorder (PD). Additionally, recent studies have shown that glutamatergic antagonist may have potential anxiolytic action.

These experimental and clinical evidences might indicate a possible use of olanzapine in the treatment for anxiety disorders.

The aim of this study is to confirm the anxiolytic and antidepressant effects of olanzapine in animals and to clarify the underlying mechanisms.

To examine this hypothesis, we used the elevated plus-maze test (EPM) either in basal condition or after restrain stress and the forced swim test (FST) in rats acutely or chronically (21 days) treated with a dose of olanzapine (0.5 mg/kg i.p.) alone or in combination with DCS, an agonist at the glycine site on NMDA receptor, or the 5-alpha-reductase inhibitor finasteride.

The results showed an anxiolytic effect of the acute but not of the chronic treatment with olanzapine. Furthermore, this anxiolytic effect was counteracted by the co-administration of olanzapine and DCS or finasteride. No antidepressant effect could be observed after either an acute or chronic treatment with olanzapine.

The anxiolytic effect of olanzapine might be due to possible actions of olanzapine on glutamatergic and corticosteroid pathways.

**IMMUNOMODULATORY EFFECT OF COMBINATION THERAPY WITH MINOCYCLINE AND ATORVASTATIN ALLEVIATES SEVERITY OF EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS**

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Experimental autoimmune encephalomyelitis (EAE), the available animal model for multiple sclerosis (MS), is a T cell-mediated autoimmune disease. Immunomodulators combined with neuroprotective drugs may represent a useful treatment in MS. We tested the effect of combined treatment of minocycline, which exhibits multiple anti-inflammatory effects with neuroprotective properties, and atorvastatin, an immunomodulator. C57Bl6/j female mice were immunized with MOG35-55 peptide emulsified in complete Freund's adjuvant. Pertussis toxin was administered i.p. Controls were injected with PBS. Immunized mice were divided into four groups treated with: minocycline (50 mg/kg i.p.), atorvastatin (1 mg/kg per os), minocycline (50 mg/kg i.p.) plus atorvastatin (1 mg/kg per os), and PBS. The mice were sacrificed 26 and 50 days post immunization (p.i.). Weight loss and clinical signs were examined daily. Anti-MOG (Myelin oligodendrocyte glycoprotein) antibody response was assessed by solid-phase ELISA (26th day p.i.). Histo- and immuno-histochemical studies were carried out on paraffin-embedded spinal cord sections. Inflammation was assessed by the presence of inflammatory infiltrates stained with hematoxylin and eosin and by the glial fibrillary acidic protein (GFAP) immunoreactivity. Axonal and neuronal pathology were assessed by Bielschowsky staining and by the antibody anti NeuN, respectively. Treatment of mice with either atorvastatin or minocycline alone did not alter disease severity until day 36 and day 46 p.i., respectively. Combined treatment, markedly ameliorated the EAE in a statistically significant way by 22 to 30 days p.i. The number per section of inflammatory infiltrates in mice sacrificed at day 26 p.i. was significantly ( $P<0.001$ ) decreased in all treated mice. The demyelination index demonstrated, at day 26 p.i, a beneficial outcome for all three treatments with a median score of 1 for the mice individually treated and 0 for mice treated with the two drugs. We have found by the stereological analysis that activated astrocytes are significantly reduced in mice treated with atorvastatin plus minocycline ( $P<0.05$ ) in comparison to the EAE-PBS mice. In the same group of treated animals neurons are increased in a statistically significant way ( $P<0.01$ ), even if a less marked increase was observed in the group treated with atorvastatin ( $P<0.05$ ). At the onset of clinical signs, we observed a significant reduction of peptide specific proliferation in all pharmacologically treated animals in comparison to the EAE-PBS treated group. Moreover combined treatment resulted in a significant reduction of MOG-antibody response ( $P<0.001$ ). In conclusion these data demonstrate the usefulness of the combined treatment in the therapy of MS.

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## MITOCHONDRIAL DYSFUNCTIONS IN A TRANSGENIC MURINE MODEL OF ALZHEIMER'S DISEASE

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Alzheimer's disease (AD) is characterized by two hallmark lesions: diffuse and neuritic plaques, which are predominantly composed of the amyloid beta ( $A\beta$ ) peptide, and neurofibrillary tangles, composed of filamentous aggregates of hyperphosphorylated tau protein. Recent studies suggested that  $A\beta$  can directly interact with mitochondria causing leakage of reactive oxygen species (1), but no evidences have been produced so far on the mechanisms involved in  $A\beta$ -induced mitochondrial dysfunction.

In the present study, we used a triple-transgenic murine model of AD (3xTg-AD), which progressively develops  $A\beta$  and tau pathology, with a temporal- and regional-specific profile that closely mimics the human pathology (2). To directly test the hypothesis of whether the regional-specific development of  $A\beta$  and tau pathologies interfere with mitochondrial respiratory chain, brain mitochondria were isolated from frontal cortex, hippocampus, striatum, and cerebellum of 18-month old 3xTg-AD and non Tg mice. The following parameters were measured: 1) state 4 and state 3 respiration rates in the presence of either Complex I or Complex II substrates; 2) respiratory control ratio (RCR); 3) membrane potential.

Results revealed that, except for the cerebellum, all the mitochondria isolated from 3xTg-AD mice and monitored in state 4 and state 3, showed an alteration in Complex I. In particular, adding glutamate/malate as substrate, higher oxygen consumption was found in state 4 vs. state 3, which accounts for a lower RCR in 3xTg-AD mice with respect to non Tg mice. When the respiratory activity of Complex II in state 4 and state 3 was monitored in mitochondria incubated with succinate as substrate and rotenone as inhibitor of Complex I, a significant increase of oxygen consumption was observed in mitochondria isolated from striatum, cortex and hippocampus of 3xTg-AD mice compared to non Tg mice. Such increase was observed both in state 4 and state 3 and the RCR resulted similar to that obtained in mitochondria of non Tg mice. Our results suggest that the mitochondria isolated from brain regions of 3xTg-AD mice displaying higher  $A\beta$  and tau lesions showed an alteration in Complex I, which might account for a mitochondrial uncoupling between respiratory chain complexes and ATP synthesis. This hypothesis is supported by a lower inner mitochondrial membrane potential found in 3xTg-AD mice compared to non Tg mice.

### References:

1. Reddy PH (2006) J Neurochem 96: 1-13.
2. Oddo S, et al. (2003) Neuron 39: 409-21.

## MEMBRANE PRTEINS INVOLVED IN FLAVONOID TRANSPORT IN ENDOTHELIAL CELLS

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Endothelial cells play a key role in the maintenance of cardiovascular homeostasis. They have multiple functions, such as the preservations of integrity of the vascular wall; moreover, they represent a functional barrier between the circulating blood and the surrounding tissues, particularly at the level of the brain, through which the transport of several substances is regulated.

A great deal of epidemiological studies has highlighted a correlation between flavonoid-enriched diets and reduced risk of cardiovascular disease, thus suggesting that the vascular endothelium might be a target of dietary flavonoids. However, their transport into endothelial cells is not adequately documented.

The aim of my research was therefore to characterise flavonoid transport into cultured endothelial cells, with emphasis on the possible role of both influx and efflux transporters, whose interaction with flavonoids has been previously documented in other experimental systems. One of them is bilitranslocase, a membrane carrier promoting the influx of various organic anions, such as bilirubin and flavonoids (1). Other are some primary active multispecific efflux transporters, acting as efflux systems for flavonoids (2).

The experimental models used in my research were cultured endothelial cells explanted from either rat or human aorta and the permanent cell line E.hy 926. The expression of the above-mentioned membrane transporters was investigated by both Western blot analysis and immunocytochemistry using specific antibodies.

A transport assay was implemented in these cell cultures using quercetin as a reference flavonoid transport substrate. Its concentration in the extracellular medium was evaluated by UV-VIS spectrophotometry.

The results obtained show that quercetin is rapidly taken up into cells by a transient concentrative mode. At the peak, the intracellular concentration is estimated to be at least 10-fold higher than in the extracellular medium. An apparent efflux follows at later times. The uptake is strongly bilitranslocase-dependent, since it is inhibited by anti-bilitranslocase antibodies as well as by bilitranslocase substrates co-administered with quercetin. Notable is the strong inhibition yielded by nM concentrations of bilirubin and  $\mu$ M concentrations of the phthalein BSP(bromo sulfo phalein). The uptake appears to be the net result of both influx and efflux. Efflux seems to be catalysed by efflux pumps, since verapamil, a substrate of various primary active transporters, strongly enhanced quercetin uptake into cells. Our data indicate that MRP1, MRP2 and BCRP, but not PgP, are expressed in the investigated cells and might therefore be involved in quercetin efflux from the cells.

These results suggest that flavonoids might be bioavailable in the vascular endothelium, an essential step to understand their possible bioactivity with respect to cardiovascular diseases.

### References:

1. Passamonti S, et al. (2005) Food Res Int 38: 953-60.
2. Morris ME, Shuzhong Z (2006) Life Sci 78: 2116-30.

## INFLUENCE OF REDOX MODIFICATIONS ON PLATELET AGGREGATION

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Blood platelets are central to hemostasis and platelet aggregation is considered a direct index of platelet function. Sulfhydryl groups of proteins on the platelet surface are necessary for platelet aggregation and reactions involving disulfide bonds, sulfhydryl groups, and protein disulfide isomerase play important roles in platelet function (1-2).

Platelet activation by agonists like ADP or collagen leads to the disruption of specific disulfide bonds in the fibrinogen receptor, integrin  $\alpha$ IIb $\beta$ 3 (3). This disruption facilitates the binding of soluble fibrinogen, leading to the formation of a platelet aggregate. Activation of  $\alpha$ IIb $\beta$ 3 is key for normal hemostasis, but improper activation of  $\alpha$ IIb $\beta$ 3 can cause cardiovascular diseases (4).

In this study, we analyzed the effects of two reagents, NEM and DTT on platelet aggregation. NEM is a sulfhydryl reagent used to irreversibly inhibit the formation of cystine linking in proteins. On the other hand, DTT is a strong reducing agent frequently used to reduce the disulfide bonds of proteins and, more generally, to prevent intramolecular and intermolecular disulfide bonds from forming between cysteine residues of proteins. NEM was used in a range from 10  $\mu$ M to 500  $\mu$ M (final concentration), while DTT was used in a range from 1 mM to 10 mM (final concentration). The effects of these two compounds were analysed in *in vitro* tests both in human platelet rich plasma (PRP) and in human washed platelets. Platelet aggregation was performed according to Born's method (5) and induced by ADP and collagen; each aggregation rate was evaluated as an increase in light transmission.

DTT (3 mM and 10 mM) seemed to amplify platelet aggregation induced by ADP or collagen, at an agonist concentration that by itself caused only primary aggregation. However, when added in platelets, in absence of ADP or collagen, DTT (3 mM and 10 mM) induced platelet aggregation at the same rate. In contrast, NEM (100 - 500  $\mu$ M in PRP; 20 - 50 mM in washed platelets) inhibited platelet aggregation induced by an ADP or collagen concentration that by itself caused irreversible aggregation.

These results seem to confirm the hypothesis that redox modifications can influence platelet aggregation. Further studies to determine whether DTT and NEM at the same concentrations as above influence  $\alpha$ IIb $\beta$ 3 structure are in progress.

### References:

1. Essex DW, Li M, Miller A, Feinman RD (2001) *Biochemistry* 40: 6070-5.
2. Yan B, Smith JW (2000) *J Biol Chem* 275: 39964-72.
3. Essex DW, Li M (2003) *Biochemistry* 42: 129-36.
4. Jang Y, Lincoff AM, Plow EF, Topol EJ (1994) *J Am Coll Cardiol* 24: 1591-601.
5. Born GVR (1962) *Nature* 194: 927-9.

**EFFECTS OF METAL-BASED DRUGS ON AN IN VITRO SIMULATION OF THE METASTATIC PROGRESSION AND IN VIVO IN MICE WITH MAMMARY CANCER****Masi Alessia**

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Ruthenium compounds, among metal-based drugs, are emerging as promising anti-tumour and anti-metastatic agents. The aim of this study was that of evaluating the capacity of some representative compounds, namely  $\text{HIm}[\text{Ru(III)Cl}_4(\text{Im})_2]$  (KP418),  $\text{HInd}[\text{Ru(III)Cl}_4(\text{Ind})_2]$  (KP1019),  $\text{Ru(II)Cl}_2(\eta^6\text{-C}_7\text{H}_8)(\text{PTA})$  (RAPTA-T),  $[(\eta^6\text{-biphenyl}) \text{Ru(II) Cl}(\text{ethylenediamine})]$  PF6 (RM175), and  $[(\eta^6\text{-biphenyl}) \text{Os(II) Cl}(\text{ethylenediamine})]$  BF4 (AFAP51) to interfere with the metastatic progression. In vitro tests were done with the highly invasive breast cancer cell line MDA-MB-231 and the non tumorigenic mammary cell line HBL-100. The experimental procedures represent cell detachment from primary tumour (determined as resistance to trypsin cell harvesting), degradation of the extracellular matrix (determined as inhibition of gelatinases), migration (determined as haptotactic, chemotactic, and Matrigel® invasion), and adhesion to a distant organ/tissue (determined as the ability to re-adhere after treatment). KP418, KP1019, and RAPTA-T significantly reduced the number of cells recovered in 30 min from the treated plates using diluted trypsin as compared to untreated controls on the MDA-MB-231 cell line. KP418 and RAPTA-T markedly reduced the activity/production of gelatinases, in the same cell lines, whereas KP1019 was active also in the non tumorigenic HBL-100 cells. The organometallic complexes RM175 and AFAP51 inhibited gelatinases only in the non tumorigenic HBL-100 cells. RAPTA-T significantly inhibited cell migration ability, studied with properly adapted Transwell® chambers, of MDA-MB-231 cells and it similarly inhibited the capacity of these cells to adhere, particularly to fibronectin. KP1019 showed similar results although it did not distinguish between tumour and non tumorigenic cells. Indeed, the effects of KP1019 were influenced by its cytotoxicity, particularly evident 24 h after treatment. In vivo experiments showed different activities for the tested compounds, ranging from moderate inhibition of primary tumour and of lung metastasis (KP1019), to pronounced inhibition of lung metastasis and of primary tumour (RM175), to inhibition of lung metastasis (RAPTA-T) with no inhibition of primary tumour, to completely ineffectiveness (AFAP51). Globally taken, these data show that a compound such as KP1019, although being very closed to the selective anti-metastatic agent  $\text{HIm}[\text{Ru(III)Cl}_4(\text{ImDMSO})]$  (NAMI-A), is completely free of selective anti-metastatic effects either in vitro or in vivo. A similar discrepancy is also evident for RAPTA-T (active) and RM175 (inactive), two organometallic ruthenium drugs, structurally very similar, suggesting that the interaction of metal-based drugs with the metastatic process implies the targeting of very specific and selective molecules displayed preferentially (or only) by the invasive and metastatic cells.

**PRECISION-CUT LIVER SLICES METHOD TO STUDY L-DEPRENYL METABOLISM****Materozzi Giada**

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Precision-cut liver slices are a widely accepted *in vitro* system for the examination of drug metabolism, enzyme induction, and hepatotoxic effects of xenobiotics. Earlier investigations in our laboratory focused on setting up the preparation of precision-cut slices from rat liver and their maintenance in culture. We prepared slices that retained good cell-viability for 24 h of incubation (as measured by glutathione content and LDH leakage). These slices possessed relatively stable metabolic functions, as demonstrated by the time dependent-metabolism of 7-ethoxycoumarin. The aim of this investigation was the metabolic profile analysis of the well-known drug, L-deprenyl, using the precision-cut liver slices model.

Precision-cut rat liver slices were prepared from tissue core (8 mm) using a Krumdieck tissue slicer filled with oxygenated, ice-cold Krebs-Henseleit buffer. After a pre-incubation period of 30 min, slices were individually incubated in RPMI1640 complete medium under carbogen atmosphere and incubations were carried out at 37°C in 12 wells plates with a continuous gentle shaking (1). The incubation medium was supplemented with 100 µM L-deprenyl for the corresponding metabolism studies. After various time of incubation (2, 4, 6, and 24 h), medium samples were taken and the formation of L-deprenyl metabolites was determined by GLC (2).

The analysis at different time of incubation of L-deprenyl metabolism showed a marked decrease of the compound and an evident increase of its metabolites, nordeprenyl, methamphetamine and amphetamine, following 2 h of incubation. The kinetic study of nordeprenyl and methamphetamine formation showed an atypical Michaelis-Menten kinetics. These results were confirmed by the Eadie-Hofstee representation. The intrinsic clearance determination suggested that the enzyme involved in nordeprenyl and methamphetamine formation should have an auto-activating process. The kinetic analysis performed in microsomal fractions of rat liver revealed, for nordeprenyl, the same atypical Michaelis-Menten kinetics shown in the slice experiments. However, methamphetamine formation in microsomes possessed biphasic Michaelis-Menten kinetics as confirmed by the Eadie-Hofstee plot. These kinetic parameters underlined the presence of a high affinity component and a low affinity component.

The  $K_m$  values obtained in the precision-cut liver slices model were of the same order of magnitude as those observed with rat microsomes for both nordeprenyl and methamphetamine. However, the  $V_{max}$  was 3-fold higher in microsomes, probably due to the lower cytochrome p450 content in the slices. Moreover the different kinetic parameters of methamphetamine in the precision-cut liver slices compare to microsomes could be the results of the complex multi-enzymatic system present in the slices that could interact with L-deprenyl, modifying its concentration in the enzymatic site.

In conclusion, the precision-cut liver slices represent an *in vitro* model alternative to microsomes for studying drug metabolism. Further studies are necessary to clarify the differences between the two systems.

## References:

1. Hashemi E, Dobrota M, Till C, Ioannides C (1999) *Xenobiotica* 29: 11-25.
2. Dragoni S, et al. (2003) *J Neurochem* 86: 1174-80.

**ADULT NEURAL STEM CELLS OF THE SUBVENTRICULAR ZONE EXPRESS THE RECEPTOR FOR ADVANCED GLYCATED END-PRODUCTS****Meneghini Vasco**

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The receptor for advanced glycated end-products (RAGE) is a member of the immunoglobulin superfamily of cell surface protein that interacts with a range of ligands involved in physiological and pathological processes in the CNS, such as angiogenesis, neuronal migration and differentiation, age-related neurodegeneration, inflammation, neurodegenerative disease and affective disorders. Very little is known about the involvement of RAGE in adult neurogenesis, except that the receptor is expressed in the actively proliferating progenitor cells of the hippocampal subgranular zone (SGZ) (1). Our group has provided evidence for the selective expression of members of the NF- $\kappa$ B family in the BrdU-positive cells of the neurogenic areas of adult mouse brain (2). Interestingly, these transcription factors are usually involved in an autoregulatory positive feedback loop whereby RAGE itself is up-regulated *via* the presence of its ligands (3). Based on these observations, we decided to investigate the possible contribution of the RAGE/NF- $\kappa$ B pathway in the modulation of adult neurogenesis.

For this purpose we have isolated and expanded *in vitro* the multipotent neuronal progenitor population of adult mouse subventricular zone (SVZ) using the neurosphere assay.

By immunofluorescence analysis, we demonstrated that RAGE is highly expressed in the membrane of undifferentiated nestin-positive neuronal progenitors and that the NF- $\kappa$ B family member p50 is expressed in a neurosphere cell subpopulation. Surprisingly, the other major protein member of the NF- $\kappa$ B family, RelA/p65, was not expressed in this cell culture model. The results were further confirmed by Western blot analysis, which allowed dissecting the different RAGE isoforms expressed in neurosphere cells. At present, we are investigating the possible role of the RAGE/ NF- $\kappa$ B axis in the modulation of proliferation, migration and differentiation of neuronal progenitors by exposing neural stem cells to RAGE ligands including HMGB-1, S100 $\beta$ , AGE-BSA and  $\beta$ -amyloid protein.

In summary, our preliminary observation of the presence of RAGE in the membrane of the SVZ multipotent neuronal progenitor population opens new interesting questions about the role of the RAGE/ NF- $\kappa$ B axis in regulating neural stem cell function in physiology and pathology.

## References:

1. Manev H, Uz T, Manev R (2003) J Aff Dis 75: 59-64.
2. Denis-Donini S, Caprini A, Frassoni C, Grilli M (2005) Dev Brain Res 154: 81-9.
3. Bierhaus A, et al. (2005) J Mol Med 83: 876-86.

**GUANOSINE INHIBITS BETA-AMYLOID PROTEIN-INDUCED APOPTOSIS BY UPREGULATION OF PROTEASOME ACTIVITY IN HUMAN NEURONAL CELLS****Merlicco Adriana**

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Neurodegeneration in Alzheimer's disease (AD) is associated with abnormal accumulation of neurotoxic  $\beta$ -amyloid (Abeta) protein, which causes apoptosis of neuronal cells (1). The accumulation and aggregation of the abnormal proteins suggests an impairment of the ubiquitin-dependent degradation pathways. In particular, the proteasome might serve as a preferential target for pathogens, which could induce molecular changes such as mutations or conformational alterations causing the proteasome to fail (2). A recent study also shows that the proteasome is implicated in the degradation of protein controlling apoptosis (3). Guanosine (GUA) and other nonadenin-based purines have many neurotrophic and neuroprotective effects, such as promotion of neurite outgrowth, increased release of NGF and protection of astrocytes against apoptosis induced by staurosporine (4). However, the role of GUA in AD is still not well established. In this study, we investigated the neuroprotective effects of GUA against Abeta protein-induced apoptosis in human neuronal SH-SY5Y cells. The apoptosis in term of mitochondrial function loss and translocation of phosphatidylserine (PS) was induced by 3 h treatment of SH-SY5Y cells with 1  $\mu$ M of Abeta peptide (25-35), a neurotoxic core of Abeta protein. Treatment of SH-SY5Y cells with GUA (12.5-75  $\mu$ M) in presence of Abeta (25-35) showed a strong dose-dependent inhibitory effects on Abeta (25-35) induced apoptotic events. The maximum inhibition of mitochondrial function loss (66%) and translocation of PS (64%) was observed with 75  $\mu$ M GUA. Subsequently, to investigate whether neuroprotection of GUA can be ascribed to its ability to increase heat shock proteins (HSP) and proteasome activity levels, we used KNK437 and lactacystin, specific inhibitors of HSP70 and proteasome, respectively. We found that the antiapoptotic effects of GUA were abolished by lactacystin but not KNK437. Interestingly, the treatment of SH-SY5Y with 75  $\mu$ M GUA induced a strong increase of proteasome activity (170%). In parallel, no increase of ubiquitinated protein levels was observed at similar experimental conditions adopted. Taken together, these results demonstrate that GUA neuroprotective effects against Abeta-induced apoptosis are mediated, at least in part, via proteasome activation. In particular, these findings suggest a novel neuroprotective pathway mediated by GUA, which involves a rapid degradation of proapoptotic proteins by the proteasome. Further studies will be required to elucidate its cellular and molecular mechanisms at neuronal level. In conclusion, these findings suggest a role for GUA as a potential drug in the treatment of AD.

## References:

1. Mattson MP (2000) *Nature Rev Mol Cell Biol* 1: 120-9.
2. Schmitt HP (2006) *Med Hypotheses* 67: 311-7.
3. Favit A (2000) *J Neurochem* 75: 1258-63.
4. Ciccarelli R (2001) *Int J Neuroscience* 19: 395-414.

**PRECLINICAL STUDIES OF COMBINATION OF GEMCITABINE, PEMETREXED AND ZD6474 AND PHARMACOGENETIC EVALUATION OF RESPONSE TO GEMCITABINE IN BLADDER TRANSITIONAL CELL CANCER****Mey Valentina***Dipartimento di Medicina Interna, Divisione di Farmacologia e Chemioterapia, Università degli Studi di Pisa.**E-mail: valmey@gmail.com*

The deoxycytidine analogue gemcitabine is an active agent against a variety of solid tumours, including genito-urinary malignancies. However, standard treatments have modest effect against bladder cancer and a great deal of interest has been focused on research into new drugs and new drug combinations for chemotherapy of these diseases. In particular, several studies are investigating the emerging role of pemetrexed (multitargeted antifolate, MTA) and the biological agents targeting molecular pathways involved in tumour growth and angiogenesis, such as ZD6474, a new inhibitor of the kinase domain-containing of VEGFR2 and EGFR. Therefore, the present studies were performed to investigate the capability of gemcitabine, pemetrexed or ZD6474 to synergistically interact in T24 and J82 bladder cancer cells, and to establish a correlation between drug activity and gene expression of selected genes in tumour samples. Cells were treated with gemcitabine, pemetrexed or ZD6474, alone or in sequence. Pharmacologic interaction was studied using the combination index (CI) method. The effects of drugs on cell cycle, Akt (S473), EGFR (Y992 and Y1173) phosphorylation and apoptosis were investigated by flow cytometry, ELISA, and fluorescence microscopy. Finally, quantitative PCR was used to study target gene expression profile and its modulation by single drugs. Therefore, we investigated the relationship between the gene expression levels of enzymes involved in gemcitabine uptake and activation in TCC tissues and pathological response to treatment with gemcitabine. The interaction between gemcitabine and pemetrexed or ZD6474 was synergistic; indeed, pemetrexed favoured gemcitabine cytotoxicity by increasing cellular population in S-phase, reducing Akt phosphorylation as well as by inducing the expression of the major uptake system of gemcitabine, the human equilibrative nucleoside transporter-1 (hENT1), and of the gemcitabine activating enzyme deoxycytidine kinase (dCK) in both cell lines. ZD6474 significantly decreased the amount of activated Akt and cells exposed to both drugs presented typical apoptotic morphology. In particular, drug combinations increased apoptotic cell death with respect to control cells ( $P < 0.05$ ). Furthermore, gemcitabine significantly enhanced the content of pY1173 EGFR levels, while ZD6474 decreased EGFR phosphorylation at both tyrosine residues. PCR analysis showed that ZD6474 increased the gene expression ratio between dCK and the gemcitabine target ribonucleotide reductase (RR) in pancreatic cell lines. The pharmacogenetics determinants of gemcitabine were also studied in 22 bladder cancer patients (4 with pathological remission and 18 with stable disease). Patients who achieved a pathological complete response to intravesical gemcitabine showed higher expression levels of dCK (median value 1.026) and lower levels of RRM2 (median value 0.820). Moreover, the calculated  $dCK \times hENT/RR$  expression ratio resulted as a potential pharmacogenetic determinant predictive of pathological response ( $P = 0.012$ ). These data provide evidence that the combination of gemcitabine with the new drugs pemetrexed and ZD6474 is synergistic against bladder cancer cells and that the assessment of the expression of genes involved in gemcitabine uptake and activation might represent a new tool for treatment optimization of bladder cancer patients.



## AN EXPERIMENTAL MODEL FOR NATURALLY OCCURRING PEYRONIE'S-LIKE DISEASE

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Peyronie's disease (PD) is a connective tissue disorder involving the growth of fibrous plaques of *tunica albuginea* of the penis with curvature and distortion, usually during erection, penile pain and erectile dysfunction (1). The disorder is a localised, fibrosing condition of the penis that occurs in up to 9% of men. PD is a poorly understood disease on molecular and physiopathological basis. Although its aetiology has not been elucidated, PD probably results from the presence of a predisposing genetic susceptibility combined with an inciting event, most probably trauma. There is only one animal model developed in 1997 (2) realized by injection of transforming growth factor- $\beta$  (TGF- $\beta$ ) into the penis tunica of rats. TGF- $\beta$  has been implicated in many chronic fibrotic conditions: alterations in TGF- $\beta$  pathway are a potential factor in the pathogenesis of PD (3). *Tight skin* mouse (*Tsk*) has been proposed as an experimental model for disease coupled with abnormalities of the connective tissue (4). Indeed the scope of our study was to characterize and propose the mouse strain *Tsk* as a new model of PD naturally occurring.

*Tsk* mice and their background C576B16J have been followed from 2 up to 12 months. Morphological appearance of the penis was monitored at different ages. Histological and immunohistochemical studies were used to characterize the disease. Collagen was evaluated by hydroxyproline assay and by Masson's trichrome reaction. TGF- $\beta$  and iNOS levels were monitored by quantitative RT-PCR.

The outcomes of our studies report that *Tsk* develops an age dependent PD-like disease. The fibrosis becomes more evident during the months reaching its maximum at 12 months. Histological studies pointed up the presence of an evident structural disorder combined with thickening of the tunica and TGF- $\beta$  staining around the small vessels. Hydroxyproline assay demonstrated that collagen deposition increases over time reaching its maximum at 12 months. Quantitative RT-PCR for TGF- $\beta$  message increases within the first 8 months and then starts declining. Contrarily the mRNA copies for iNOS increases up to 12 months.

*Tsk* mice can represent a model of particular value for investigating on the molecular mechanism involved in the pathogenesis of PD and for studying new therapeutic approaches.

### References:

1. Gelbard MK, Dorey F, James J (1990) J Urol 144: 1376-9.
2. El-Sakka AI, Hassoba HM, Pillarisetty RJ, Dahiya R, Lue TF (1997) J Urol 158: 1391-4.
3. Haag SM, et al. (2007) Eur Urol 51: 255-61.
4. Saito S, Pasturi K, Bona C (1999) Curr Rheumatol Rep 1: 34-7.

## PROTEIN KINASE C DELTA INHIBITION PROMOTES ENDOTHELIAL CELL SURVIVAL

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Ischemic/reperfusion damage, occurring after myocardial ischemia, impairs endothelial cell functions due to blood flow reduction and the following reactive oxygen species (ROS) production. The increased level of ROS is responsible for the protein kinase C  $\delta$  (PKC $\delta$ ) activation. Recent studies demonstrate that the inhibition of PKC $\delta$  translocation through the administration of a new selective peptide  $\delta$ V1-1, exerts a cardioprotective effect in heart ischemia inhibiting PKC $\delta$ -induced apoptotic cascade in myocyte.

The aim of this study was to test the role of  $\delta$ V1-1 on ROS-induced endothelial cell dysfunction.

$\delta$ V1-1 activity was assessed on a model of coronary microvascular endothelial cells (CVEC). Production of ROS was obtained by culturing endothelial cells for 24 h in low serum concentration (0.1% CS). Cell survival in the presence or in the absence of PKC $\delta$  inhibitor was assayed by cell proliferation.

Low serum concentration (0.1%) promoted ROS production in endothelial cells. ROS induced PKC $\delta$  translocation and reduced microvascular endothelial cell proliferation. PKC $\delta$  inhibitor,  $\delta$ V1-1, suppressed ROS-induced PKC $\delta$  translocation and rescued endothelial cell proliferation.

The data demonstrate that, in endothelial cells, oxidative stress promotes ROS production. Increased ROS led to PKC $\delta$  translocation and is associated with reduced cell growth. The PKC $\delta$  selective inhibitor  $\delta$ V1-1, exerts a protective effect through the inhibition of ROS production.

**CELLULAR AND MOLECULAR MECHANISMS FOR THE SYNERGISTIC CYTOTOXICITY ELICITED BY OXALIPLATIN AND PEMETREXED IN COLON CANCER CELL LINES****Nannizzi Sara***Divisione di Farmacologia e Chemioterapia, Dipartimento di Medicina Interna, Università degli Studi di Pisa, via Roma 55, 56126 Pisa**E-mail: snannizzi@gmail.com*

Oxaliplatin is active in the treatment of colorectal cancer and its effect is improved upon combination with thymidylate synthase (TS) inhibitors. Since the novel folate analogue pemetrexed blocks folate metabolism and DNA synthesis by inhibiting TS, dihydrofolate reductase (DHFR) and glycinamide ribonucleotide formyltransferase (GARFT), the present study investigated the *in vitro* cytotoxicity of oxaliplatin and pemetrexed combinations. Human HT29, WiDr, SW620 and LS174T colon cancer cells were treated with oxaliplatin and pemetrexed, alone or in combination. Drug interaction was studied using the combination index method, while cell cycle was investigated with flow cytometry. Moreover, the effects of drugs on Akt phosphorylation and apoptosis were studied with ELISA and fluorescence microscopy, respectively. RT-PCR analysis was performed to assess whether oxaliplatin and pemetrexed modulated the expression of pemetrexed targets, folypolyglutamate synthase (FPGS) and excision repair cross complementing group 1 and 2 (ERCC1/2). Finally, the basal gene expression analyses of single, i.e. TS, DHFR, GARFT and FPGS or combined genes, i.e. FPGS/(TS\*DHFR\*GARFT) were related to pemetrexed chemosensitivity. A dose-dependent inhibition of cell growth was observed after oxaliplatin and pemetrexed exposure. A synergistic interaction was observed with simultaneous and sequential combinations. Oxaliplatin significantly enhanced cellular population in the S phase in HT29 (from 26.2% to 40.9%), SW620 (from 44.9% to 57.1%) and LS174T (from 33.5% to 50.0%). Drug combinations significantly increased apoptotic index with respect to single agents. Oxaliplatin and pemetrexed were able to reduce the phosphorylated Akt in all cell lines ( $P < 0.05$ ), with pemetrexed being more potent than oxaliplatin. Finally, RT-PCR showed that pemetrexed significantly reduced ERCC1 gene expression in HT29 and LS174T cell lines. Similar results were observed for ERCC2, whose expression was decreased by pemetrexed up to  $-59.8 \pm 5.0\%$  in HT29 cells,  $-36.8 \pm 3.0\%$  in WiDr cells and  $-22.6 \pm 7.4\%$  in SW620 cells. Basal mRNA levels of FPGS, TS, DHFR and GARFT were analyzed and related to pemetrexed sensitivity: a good correlation was found between the FPGS/(TS\*DHFR\*GARFT) ratio and  $IC_{50}$  values of pemetrexed ( $r^2 = 0.954$ ;  $P = 0.024$ ). Moreover, oxaliplatin modulated gene expression of pemetrexed target enzymes in WiDr, SW620 and LS174T cells ( $P < 0.05$ ). TS expression was significantly decreased up to  $-25.7 \pm 4.0\%$  and  $-39.0 \pm 6.0\%$  in SW620 and LS174T cells, respectively, while DHFR and GARFT expression was strongly reduced in LS174T cells ( $-78.5 \pm 3.5\%$  and  $-72.2 \pm 4.4\%$ , respectively) and slightly modulated in WiDr and SW620 cells. These data demonstrate that oxaliplatin and pemetrexed synergistically interact against colon cancer cells, through modulation of cell cycle, inhibition of Akt phosphorylation, induction of apoptosis and modulation of gene expression.

**S 18886, A THROMBOXANE A2 RECEPTOR ANTAGONIST, PREVENTS OCCURRENCE OF SPONTANEOUS BRAIN DAMAGE IN STROKE-PRONE RATS VIA ANTI-INFLAMMATORY ACTIVITIES**

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Spontaneously hypertensive stroke-prone rats (SHR-SP) are an established model of human cerebrovascular disease. In this rat strain, the development of hypertension and inflammation precedes the appearance of brain abnormalities. The aim of the present investigation was to assess the efficacy of S 18886, an orally active antagonist of TP-receptors (the receptors for thromboxane A<sub>2</sub>), in protecting the brain of SHR-SP and whether this effect was related to its anti-inflammatory properties. Male SHR-SP (n=10 per group), fed with a high-salt diet, received by gavage vehicle or S 18886 (3 or 30 mg/kg/day). In vehicle-treated animals, brain lesions, as detected by magnetic resonance imaging, developed spontaneously after 40±2 days (mean±SEM). Treatment with S 18886 had no effect on arterial blood pressure, significantly delayed the appearance of brain damage, at the dose of 30 mg/kg/d (P<0.001), and increased survival, in a dose dependent manner (P<0.001 and P<0.0001 at the dose of 3 and 30 mg/kg/day, respectively). In comparison with vehicle-treated SHR-SP, treatment with S 18886 (30 mg/kg/day; n=5) preserved brain tissue by preventing macrophage infiltration (ED1 positive cells) (P<0.05), and reduced the accumulation of perivascular macrophages (ED2 positive cells) and lymphocytes T helper (CD4<sup>+</sup> positive cells) as assessed by immunohistochemistry. Furthermore, S 18886 attenuated the transcription of the pro-inflammatory cytokines IL-1β, TNF-α, IL-6, and MCP-1, as assessed by RT-PCR.

These data indicate that S 18886 prevents the occurrence of spontaneous brain damage in SHR-SP by reducing inflammation, suggesting that S 18886 may exert a beneficial anti-inflammatory effect in cerebrovascular disease.

**ROLE OF NOCICEPTIN AND ITS RECEPTOR IN ANIMAL MODEL OF ASTHMA****Orlotti Donatella**

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Tachykinergic neurotransmission is modulated by an endogenous peptide nociceptin/orphanin FQ (N/OFQ), via selective activation of N/OFQ peptide (NOP) receptor. The peptide/receptor system is considered a “non- opioid branch of the opioid family” of peptides and receptors, because the N/OFQ–NOP receptor and classical opioid have structural and transductional similarities, but pharmacological and functional differences. Some studies have been showed that N/OFQ-NOP receptor may influence airway physiology by modulating tachykinergic neurotransmission. In this study, we have been evaluated the role of NOP receptor activation in bronchoconstriction induced by capsaicin in isolated and perfused mouse lungs and verified the ability of N/OFQ to protect from experimental-induced allergic asthma in a group of animal sensitised to ovalbumine. We have also quantified endogen nociceptin before and after capsaicin, to verify a capsaicin-dependent modulation of N/OFQ.

In wild type and knockout NOP<sup>-/-</sup> mice we have been studied the responsiveness to capsaicin, evaluating bronchopulmonary function (total lung resistance  $R_L$  and dynamic compliance  $C_{dyn}$ ). Moreover to confirm a protective action of nociceptin-NOP receptor system in a model of allergic asthma, we evaluated the bronchopulmonary function also in a group of animal sensitised to ovalbumine.

Capsaicin led to a significant increase in bronchoconstriction in terms of  $R_L$  increase in wild type and this effect was higher both in knockout and sensitised mice. In wild type mice this effect was inhibited by pre-treatment with N/OFQ or NOP receptor agonist UFP 112, which mimicks the inhibitory effect of N/OFQ, being 10-fold more potent. Pretreatment with UFP 101, a selective NOP receptor antagonist, blocked the effects of both agonists. In knockout mice, N/OFQ and UFP 112 was unable to modify the capsaicin-induced bronchoconstriction, while the effects of both NOP agonists on sensitised mice were reduced. Perfusate analysis showed an increase of this endogen substance after administration of capsaicin in wild type mice, and a decrease of nociceptin in sensitized mice.

These results show that bronchoconstriction induced by capsaicin is inhibited by stimulation of NOP receptor. Moreover, the perfusate data suggest a neuromodulatory effect of N/OFQ on capsaicin-induced release of tachykinin from capsaicin-sensitive sensory nerve endings.

## MESENCHYMAL STEM CELLS-BASED IMMUNOTHERAPY IN PANCREATIC ISLET TRANSPLANTATION

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Insulin-dependent diabetes mellitus (IDDM) or Type I diabetes is an autoimmune disease that causes the degeneration of pancreatic beta cells. The main therapeutic option for IDDM is the substitutive therapy with insulin, which prevents ketoacidosis and diabetic coma. However, the lack of the negative feedback between plasma glucose and insulin release, in the long-term may lead to severe vascular and neurological complications. A cure for IDDM requires whole-pancreas or islet transplantation, but these therapeutic options have met with problems too. In addition to the usual problems with organ or tissue transplantation, i.e. limited availability of viable organs and the known side-effects associated with life-long immunosuppressive therapy, there are three additional problems specific for whole-pancreas or islet transplantation. The first problem is the limited efficacy of current immunosuppressive protocols against the autoimmune response to islet antigens, such as the 65 kDa isoform of the enzyme glutamic acid decarboxylase (GAD 65). The second problem is the beta cells toxicity of some immunosuppressive drugs, such as cyclosporin A and FK-506. The third problem is the recurrence of the autoimmune response against islet antigens. Several recent reports indicate that mesenchymal stem cells (MSCs) may modulate the immune response by releasing several cytokines, both *in vitro* (1) and *in vivo* (2). The main aims of the present work were to: evaluate MSCs as immunosuppressive agents in islet cells transplantation; compare the immunosuppressive efficacies of MSCs and conventional immunotherapy; evaluate MSCs effects on plasma glucose levels after islet transplantation.

Seven hundred or 1,400 islet equivalents (IE) isolated from rat pancreata were transplanted into the portal vein of recipient diabetic rats (plasma glucose higher than 400 mg/dl) after 5 days from a single high dose (65 mg/kg) of streptozotocin (STZ). In preliminary experiments islet localization in the liver was confirmed by nuclear magnetic resonance imaging of islet labelled by the super paramagnetic contrast agent Resovist. In a first series of experiments 700 IE from Sprague-Dawley were transplanted to diabetic Wistar recipient alone or in combination with MSCs. Two-way ANOVA on repeated measurements for the effect of time and treatment, followed by Tukey's tests, indicated a statistically-significant reduction of plasma glucose in rats receiving 700 IE into the portal vein plus 150,000 MSCs via the tail vein on the transplantation day compared to rats receiving 700 IE alone. Similar effects were observed in rats receiving additional 150,000 MSCs injections on the 2<sup>nd</sup> and 4<sup>th</sup> day after transplantation. Control experiments did not reveal a significant reduction in plasma glucose in rats receiving 3x150,000 injections of MSCs alone. The improvement in glycaemic control was paralleled by a significant reduction in lymphocyte infiltration of hepatic blood vessels and tissue, confirming the immunosuppressive action of MSCs. We next addressed the ability of MSCs to work against auto and alloantigens by transplanting inbred Lewis diabetic rats with 700 IE from either inbred Lewis or inbred Wistar-Furth rats, respectively. In both models, a significant reduction of plasma glucose was measured in rats receiving 700 IE plus 3x150,000 MSCs injections. Furthermore, in both models, the overall effects of MSCs were comparable to those of immunotherapy with a combination of FK-506 (Tacrolimus), rapamycin (Sirolimus), and a monoclonal antibody (Simulect) to the IL-2 receptor.

These data provide the first evidence on the efficacy of MSCs as immunosuppressive agents in pancreatic islet transplantation. An intriguing possibility is that MSCs do not just suppress the immune response of the recipient against transplanted antigens; they may in addition down regulate the expression of autoantigens, such as GAD 65, by the transplanted beta cells (3). Future work will explore the effects of coapplication of conventional immunotherapy and MSCs on graft survival and plasma glucose in transplanted animals.

### References:

1. Aggarwal S, Pittenger MF (2005) *Blood* 105: 1815-22.
2. Le Blanc K, et al. (2004) *Lancet* 363: 1439-41.
3. Hao W, Palmer JP (1995) *Autoimmunity* 22: 209-18.

**INFLUENCE OF LIVER FUNCTIONAL STATUS ON THE PHARMACOKINETIC INTERACTION BETWEEN THE CYP3A4 INHIBITOR ERYTHROMYCIN AND QUININE**

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*In vivo* inhibition of cytochrome P450 (CYP) 1A2 by the reversible inhibitor fluvoxamine causes a reduction in the clearance of the CYP1A2 substrates lidocaine and theophylline, which decreases in proportion to the degree of liver dysfunction (1-2). The objective of this study was to evaluate the effect of liver cirrhosis on the inhibition of the metabolic disposition of quinine, a probe of CYP3A4 (3), by the mechanism-based inhibitor erythromycin, to assess whether decreased sensitivity to metabolic inhibition in liver disease is a general characteristic, regardless of the mechanism of inhibition.

The study was carried out in 10 healthy volunteers and 20 cirrhotic patients, 10 with mild (Child grade A) and 10 with severe (Child grade C) liver dysfunction, according to a randomized, double-blind, 2-phase, crossover design. In one phase all participants received placebo for 5 days; in the other phase they received three 600-mg erythromycin ethylsuccinate doses, 8 h apart, for 5 days. On day 2, quinine sulphate (500 mg) was administered orally 1 h after the morning erythromycin dose. Concentrations of quinine and its metabolite 3-OH-quinine were measured in plasma and urine up to 96 h.

The effects of erythromycin co-administration were dependent on liver function. Inhibition of quinine clearance decreased from about 33% in healthy subjects, to 29% in patients with mild liver dysfunction, with proportional increases in terminal half-lives, whereas virtually no effect was produced in patients with severe liver dysfunction. At variance with quinine, erythromycin co-administration reduced the formation clearance of 3-OH-quinine to the same extent (60 to 70%) in all three studied groups. Renal clearance of 3-OH-quinine was also inhibited by erythromycin.

The effect of liver dysfunction on the inhibition of CYP-mediated drug metabolism is a general phenomenon, independent of the type of CYP involved and the mechanism of the metabolic inhibitor. Therefore, for any CYP substrate, the clinical consequences of enzyme inhibition are expected to become less and less important as liver function worsens.

The significant inhibition of the formation clearance ( $CL_f$ ) of 3-OH-quinine in Child C cirrhotics is clearly inconsistent with the lack of inhibition of systemic quinine clearance in these patients. Therefore, contrary to general beliefs, quinine 3-hydroxylation, as currently determined, cannot be used as a biomarker reaction for the activity of CYP3A4 in man.

References:

1. Orlando R, et al. (2004) Clin Pharmacol Ther 75: 80-8.
2. Orlando R, et al. (2006) Clin Pharmacol Ther 79: 489-99.
3. Wanwimorluk S, Paine MF, Pusek SN, Watkins PB (2002) Br J Clin Pharmacol 54: 643-51.

**ESTROGEN RECEPTORS CONTROL VASCULAR RESPONSES TO INFLAMMATORY CYTOKINES IN INSULIN-DEFICIENT DIABETIC MICE****Pelosi Valeria**

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The ovarian hormone 17 $\beta$ -estradiol acts predominantly via two distinct nuclear estrogen receptor (ER) isoforms, ER $\alpha$  and ER $\beta$ . They are expressed in a tissue-specific manner and control distinct biological activities. Both ER isoforms appear to mediate anti-inflammatory effects in a variety of tissues including the vascular wall (1). Estrogen is known to modulate glucose metabolism and to exert pancreatic  $\beta$ -cell protection (2), although the signalling pathways responsible for those effects are not completely understood. Because 17 $\beta$ -estradiol is a non-selective agonist of ER $\alpha$  and ER $\beta$ , we investigated the role of individual ER isoforms in the development of hyperglycaemia and vascular inflammation associated with insulin deficiency using both mice with targeted deletion of ER $\alpha$  or ER $\beta$  and ER-selective agonists. Fasting serum glucose levels were higher in ER $\alpha^{-/-}$  compared with ER $\beta^{-/-}$  or wild-type (WT) mice. Diabetes was induced in 8-week-old mice by a single i.p. injection of streptozotocin (STZ, 150 mg/kg). Despite similar worsening of glycaemic control, exposure to STZ caused significantly greater mortality in ER $\beta^{-/-}$  (41.2%) than in ER $\alpha^{-/-}$  (11.6%) or WT (14.5%) mice. Vascular biology studies were performed in cultured aortic rings isolated from WT and ER-knockout mice. Aortic rings were stimulated with a cytokine mixture comprising TNF- $\alpha$ , interleukin-1 $\beta$  and interferon- $\gamma$  for 24 h in the presence or absence of test compounds. Treatment of isolated mouse aortic rings with 1 nM 17 $\beta$ -estradiol for 24 h attenuated cytokine-driven formation of inducible NO synthase (iNOS) as detected by Western blotting. This effect was shared by the ER $\alpha$ -selective agonist 4,4',4''-(4-propyl-[1H]pyrazole-1,3,5-triyl)tris-phenol (PPT) but not by the ER $\beta$ -selective agonist 2,3-bis(4-hydroxyphenyl)-propionitrile (DPN). Accordingly, 17 $\beta$ -estradiol reduced iNOS formation in aortic rings from ER $\beta^{-/-}$  but not ER $\alpha^{-/-}$  mice. In contrast to aortic rings, addition of the ER $\alpha$ -selective agonist PPT (1 nM to 1  $\mu$ M) to rat vascular smooth muscle cells (SMC) reduced cytokine-mediated iNOS expression in cells from control but not those from diabetic animals. On the contrary, treatment with the ER $\beta$ -selective agonist DPN concentration-dependently enhanced iNOS production in both SMC groups (n=4, P<0.01, ANOVA). The activity of iNOS in response to both agonists, as determined by the Griess' reaction, reflected the above-mentioned pattern of protein synthesis. To sum up, combined use of ER-knockout mice and ER-selective agents provided evidence for an anti-inflammatory role for ER $\alpha$  as well as a potential pro-inflammatory role for ER $\beta$  in the arterial wall. Cellular and animal experiments indicated that ER $\alpha$  and ER $\beta$  play a relevant role in the control of glucose metabolism, vascular inflammatory responses and diabetes progression in rodents. Based on the present findings, ER $\alpha$ -selective activation may represent a promising pharmacologic approach to the therapeutic modulation of diabetic vascular dysfunction.

## References:

1. Mendelsohn ME, Karas RH (2005) Science 308: 1583-7.
2. Barros RP, Machado UF, Gustafsson JÅ (2006) Trends Mol Med 12: 425-31.



## IN VIVO ANALYSIS OF MITOFUSIN FUNCTION

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Charcot-Marie-Tooth disease (CMT) comprises a frequently occurring, genetically heterogeneous group of peripheral neuropathies. CMT falls into two main forms: the demyelinating CMT type 1 with decreased nerve conduction velocities and the axonal form, CMT type 2. In contrast to the well-known molecular genetic defects causing the CMT1 phenotype, the genes associated with CMT2 have only recently been identified [1].

Mutations in the mitochondrial protein mitofusin 2 (MFN2) are the most commonly identified cause of CMT2. MFN2 is ubiquitously expressed and it is localized to the outer mitochondrial membrane.

Homozygous *Mfn2* knockout mice die in midgestation owing to placental defects. Although heterozygotes were reported to have a normal phenotype, mouse embryonic fibroblast cultures from *Mfn2*-deficient mice had markedly lower mitochondrial mobility and displayed fragmented mitochondria, due to a severe reduction in mitochondrial fusion [2]. Mobility and transport of mitochondria are key elements to the functional health of the extended neuronal axons, particularly in peripheral nerves. This could be a clue to a possible mechanism of action in CMT2 [3].

To better understand the function of mitofusin as well as the mechanism responsible for the disease, we are conducting a study of the *Drosophila* homolog of mitofusin, named *dmf1*. A blast search of *Drosophila* databases using the human protein sequence, has identified a fly gene product that reveals an extensive homology with the human protein. We are using *Drosophila* as a model to conduct a detailed analysis of the loss and gain of function phenotypes, aimed at defining a functional role of this protein. These studies should help to advance our knowledge of the molecular mechanisms responsible for CMT, that are presently not understood.

### References:

- 1- Zuchner S. et al. (2004) Nat Genet 36:449-451
- 2- Chen H. et al. (2003) J Cell Biol 160:189-200.
- 3- Baloh RH. et al. (2007) J Neurosci 27:422-430.

## A PROJECT FOR MONITORING DISEASE MODIFYING DRUG PRESCRIPTIONS FOR MULTIPLE SCLEROSIS IN EMILIA ROMAGNA

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The current clinical practice for treatment of multiple sclerosis (MS) with disease-modifying drugs includes interferon- $\beta$  (IFN- $\beta$ ) and glatiramer acetate (GA), as first choice, and, in case of lack of efficacy, immunosuppressive drugs [e.g. mitoxantrone (MIT), azathioprine (AZA), methotrexate and cyclophosphamide], as well as i.v. immunoglobulins. The role of immunomodulatory agents (IFN- $\beta$  and GA) in the treatment of MS has been largely studied; however, they have some limits such as high cost and incomplete knowledge about their effectiveness and method of use, especially about the time when to start the treatment and about the co-administration with other drugs (combination therapy). Immunosuppressive drugs are very frequently used in other diseases requiring immunosuppression, but they are still under investigation for MS. A monitoring project of drug therapies to identify additional elements for the choice of these drugs and to better evaluate their comparative effectiveness has been set up in collaboration with the Multiple Sclerosis Centres of Emilia Romagna region.

A regional database of all disease modifying treatments of MS provided the following information: patient history, disease details (e.g. disability degree, relapses), drug therapies (e.g. drugs, doses, switches, side effects). One-year (May 2006 to April 2007) monitoring data were collected and analysed. For each diagnostic parameter (degree of disability, number of relapses in the 12 months following the first MS diagnosis, and results of magnetic resonance) a cut-off value was set in order to stratify the patients according to the severity of MS. Afterwards, the relationship between prescribed drug and diagnostic parameters was analysed by non-adjusted odds ratio (OR, CI 95%), setting as reference the IFN- $\beta$ 1a 30  $\mu$ g per week (the most frequently used regimen).

After one year from the project start, 12 out of 19 MS Centres were active. Data of 509 patients (71% females, 48% <40 years) were collected. The MS was diagnosed by the most recent McDonald's Criteria (49% of cases) or by the previous Poser's Criteria (41%). The relapsing remitting course was the most frequent observed (85%). The majority of patients (63%) had a quite low degree of disability [Expanded Disability Status Scale (EDSS)-score between 1 and 2.5]. IFN- $\beta$  (Avonex<sup>®</sup>, Rebif<sup>®</sup>, and Betaferon<sup>®</sup>) was prescribed in 79% of cases, followed by GA (14%) and by MIT (7%). AZA was prescribed in only 2% of cases, and other immunosuppressive agents (3 cases for methotrexate and 2 for cyclophosphamide) or i.v. immunoglobulins (a single case) in 1%. IFN- $\beta$ 1a (Avonex<sup>®</sup> and Rebif<sup>®</sup>) represented the most frequent drug choice (67%) in the relapsing remitting course patients, whereas in the progressive forms IFN- $\beta$ 1b (Betaferon<sup>®</sup>, 33%), and MIT (28%) prevailed. A statistically significant higher prevalence of use was observed for both of these drugs in comparison to Avonex<sup>®</sup> for the treatment of progressive forms. In patients with high disability (EDSS $\geq$ 3), MIT, AZA, and Betaferon<sup>®</sup> were significantly preferred to Avonex<sup>®</sup> (OR>2). High doses of IFN- $\beta$ 1a (Rebif 44<sup>®</sup>) were more used either when the cerebral magnetic resonance showed presence of active damage or when there were at least 2 relapses during the 12 months following the first MS diagnosis. The most frequent therapeutic switch occurred from IFN- $\beta$ 1a low doses (Avonex<sup>®</sup> and Rebif 22<sup>®</sup>) to IFN- $\beta$ 1a high doses (Rebif 44<sup>®</sup>), as well as from all types of IFN- $\beta$  to GA.

The database seems to be a helpful tool to monitor and to manage disease modifying therapies in MS: it could be an efficacious tool to fill the information gaps about efficacy and safety in clinical practice and those about the strategies to optimise the use of these agents. Furthermore, it could be used as starting data-set for post-marketing studies with the aim to estimate eligible patients for new therapeutic approaches, first of all for natalizumab, the newest treatment for IFN- $\beta$ -non-responder patients.

**CALCIUM/CALMODULIN-DEPENDENT PROTEIN KINASE II  
PHOSPHORYLATION MODULATES PSD-95 BINDING TO NMDA  
RECEPTOR**

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At the postsynaptic membrane of excitatory synapses, NMDA-type receptors are bound to scaffolding and signalling proteins that regulate the strength of synaptic transmission. The cytosolic tails of NR2A and NR2B subunits of NMDA receptor bind to calcium/calmodulin-dependent protein kinase II (CaMKII) and to members of the MAGUK family, such as PSD-95. Although NR2A and NR2B subunits are highly homologous, the sites of their interaction with CaMKII as well as the regulation of this binding differ.

We identified PSD-95 phosphorylation as a molecular mechanism responsible for the dynamic regulation of both PSD-95 and CaMKII interaction with NR2A subunit. CaMKII-dependent phosphorylation of PSD-95 occurs both *in vitro*, in GST-PSD-95 fusion proteins phosphorylated by purified active CaMKII, and *in vivo*, in transfected COS-7 as well as in cultured hippocampal neurons. We identified Ser73 as major phosphorylation site within the PDZ1 domain of PSD-95, as confirmed by point mutagenesis experiments and by use of a phospho-specific antibody. PSD-95 Ser73 phosphorylation causes NR2A dissociation from PSD-95, while it does not interfere with NR2B binding to PSD-95. These results identify CaMKII-dependent phosphorylation of the PDZ1 domain of PSD-95 as a mechanism regulating the signalling transduction pathway downstream NMDA receptor.

## MITHOCHONDRIAL PATHWAY OF APOPTOSIS IN THE EVOLUTIONARY PICTURE OF THE SEPSIS IN CIRRHOTIC PATIENTS

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Apoptotic processes play an important role in determining the progression of liver disease and in defense against infections. Bacterial infections are frequent in patients with cirrhosis and are associated with mortality around 50%. Innate immunity constitutes first line of defense against infections by ensuring an accurate recognition of shared pathogenic structures. Within this frame, leukocytes play a major role in the acute inflammatory response to infection.

This work aimed to examine mitochondrial apoptosis in severe bacterial infection and sepsis in cirrhotic patients.

This paper studied the mitochondrial apoptotic pathway in peripheral blood lymphocytes from 20 cirrhotic patients with sepsis (S), 30 cirrhotic patients with bacterial infection (I), 30 cirrhotic patients without bacterial infection (NI), and 10 age-matched healthy controls (C). Lymphocytes and granulocytes were isolated from the blood by means of gradient. The proteins were extracted and the expression of pro-apoptotic proteins (caspase 9 and Bax) and anti-apoptotic proteins (Bcl2, BclXL, and HSP70) was determined by Western blotting.

In lymphocytes, the expression of the proapoptotic protein Bax was increased in cirrhotics NI and I as compared to C (2- and 4-fold) and it was significantly increased in cirrhotics S as compared to healthy donors (30-fold), cirrhotics NI, and cirrhotics I (25-fold,  $P=0.003$ ).

Bax expression was increased in cirrhotic patients with bacterial infection and sepsis with Child-Pugh score B and C and with ascites ( $P=0.002$ ). Bcl 2 expression was reduced in cirrhotics I as compared to NI and it was reduced in cirrhotics S as compared to cirrhotics I and cirrhotics NI ( $P=0.04$ ). Analysis of BclXL showed a decline in cirrhotics I as compared to cirrhotics NI and a decline in cirrhotics S as compared to cirrhotics NI and I ( $P=0.01$ ). The electrophoresis profile of BclXL exhibited a greater phosphorylation in cirrhotics NI as compared to cirrhotics I and S ( $P=0.009$ ). The caspase 9 expression was increased 2-fold in cirrhotics I and 4-fold in cirrhotics S as compared to C ( $P=0.009$ ). Caspase 9 protein was highly expressed in S cirrhotics with a double increase of expression as compared to cirrhotic patients. The level of Hsp70 expression was increased in S group of patients in comparison with C, NI, and I but HSP70 expression was not changed highly ( $P=0.03$ ). In granulocytes Bax expression of cirrhotics NI, cirrhotics I, and cirrhotics S was not modified significantly as compared to healthy donors ( $P=0.03$ ); Bcl2 expression was increased in cirrhotics NI, cirrhotics I, and cirrhotics S as compared to healthy donors ( $P=0.002$ ). Bcl2 expression was highly increased in cirrhotics I as compared to cirrhotics NI and cirrhotics S (8-fold). In particular, the level of expression of Bcl 2 was increased 12-fold in cirrhotics I with Child-Pugh score A as compared to cirrhotics I with Child-Pugh score B ( $P=0.04$ ). The level of BclXL was increased 1-2 fold in cirrhotics I as compared to healthy donors and cirrhotics NI and S ( $P=0.04$ ).

The results show that alterations of expression and post-translational modifications are implicated in apoptotic regulation in bacterial infection of cirrhotic patients. These observations suggest that the analysis of cellular functions might help in anticipating prognosis in patients with cirrhosis and sepsis.

## EXPOSURE TO QT PROLONGING ANTIBACTERIAL AGENTS IN THE COMMUNITY: AN INTERNATIONAL DRUG UTILISATION STUDY

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Drug-induced long QT syndrome has received increasing regulatory attention because it is a recognized risk factor for the occurrence torsades de pointes (TdP), a potentially life-threatening ventricular arrhythmia, which can degenerate into ventricular fibrillation and sudden death.

A delay in ventricular repolarisation (leading to QT interval prolongation) is a typical effect of class III anti-arrhythmic drugs, acting through blockade of hERG K<sup>+</sup> channels. However, the growing list of non-cardiovascular drugs, such as antibacterial agents, causing unintended hERG blockade has raised concern about the risk in the general population (i.e. out-of-hospital setting).

From a pharmacological standpoint, the question is whether QT prolongation is a class effect (shared by all agents of a given pharmacological class) or it is displayed only by some compounds within a given class of non-cardiac drugs.

From a clinical standpoint, the question is whether high consumption drugs such as antibacterial agents can be ranked according to their QT prolonging potential.

Aim of this study was: 1) to classify each antibacterial agent for its QT prolonging potential according to the available information (as of December 2006) using the approach proposed by De Ponti et al. (1); 2) to verify whether the number of antibacterial agents with a QT prolonging potential has increased over the years; 3) to assess consumption of QT prolonging antibacterials (both as absolute values and as a fraction of overall antibacterial use) performing drug utilization analysis on databases of drug prescription from different European Countries; 4) to compare exposure in different European Countries in the period 1998-2006 as to evidence possible trends due to regulatory interventions.

The impact of the problem in the community will be studied considering two aspects: 1) we will apply the already developed method to rank drugs by risk of QT prolongation according to the available literature (1); 2) we will group all antibacterial QT-prolonging agents according to the relevant inclusion criteria, in increasing order of clinical relevance (Ia - cases of Torsades de pointes or ventricular tachyarrhythmia; Ib - cases of asymptomatic QT interval prolongation; IIa - hERG K<sup>+</sup> channel blockade; IIb - other pre-clinical evidences on Qt prolonging potential; III - official warnings on QT prolonging risk.

We will quantify the population exposure to antibacterials with QT prolonging potential, performing drug utilization analysis on databases of drug prescription from different Countries. The drugs meeting any inclusion criteria mentioned above will be arranged according to the ATC classification (V level) and the relevant sale data for different European Countries and for the period 1998-2006 will be collected. Moreover, for each considered Country, data of the overall consumption for each antibacterial class (ATC IV) and of the overall drug consumption will be collected. All data will be expressed as DID and PID (packages for 1000 inhabitants per day).

This study will allow analysing possible variations in exposure to QT prolonging drugs in the community because of regulatory interventions changing prescribing habits.

### References:

1. De Ponti F, Poluzzi E, Montanaro N, (2001) Eur J Clin Pharmacol 57: 185-209.

## CHRONIC THC EXPOSURE DURING ADOLESCENCE: LONG-TERM CONSEQUENCES ON THE ADULT BEHAVIOUR AND NEUROCHEMICAL CORRELATES

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Marijuana and hashish, psychoactive products of the hemp plant, are the illicit drugs most frequently used by human adolescents. Recent data estimate that the use of cannabis starts in the period from 11-12 to 17-18 years of age, although even earlier use (9-10 years) is now reported.

There are little and often contradictory studies on the long-term neurobiological consequences of cannabinoid consumption in adolescents. Adolescence represents a critical phase for cerebral development, characterized by strong neuronal plasticity, with sprouting and pruning of synapses, variations of neurotransmitter concentration and their receptor levels.

The aim of this work was to study the long-term consequences of adolescent assumption of  $\Delta^9$  tetrahydrocannabinol (THC) on mood and cognitive parameters, through behavioural and neurochemical assays. Adolescent male and female rats (35-45 Post Natal Day) have been treated with increasing doses of THC for 11 days and left undisturbed until their adulthood (75 PND) when the behavioural and neurochemical assays were performed.

Adolescent THC exposure produced different effects on male and female rats in adulthood.

The day after THC treatment (46 PND) there was significant, widespread decrease in CB1 receptor binding and functionality in different brain areas of both female and male rats. CB1 receptor level and functionality in adulthood (75 PND) were significantly reduced only in the amygdala, Ventral Tegmental Area and nucleus accumbens of female rats whereas in males significant alterations were determined in the amygdala and hippocampus.

Neither female nor male rats showed alteration in anxiety responses (elevated plus maze and open field tests) but a significant "behavioural despair" (forced swim test) paralleled by anhedonia (sucrose preference) was present in females (-40% sucrose intake). In contrast, male rats did not present behavioural despair but exhibited anhedonia.

This different behavioural picture was supported by biochemical parameters of depression, namely CampResponsiveElementBinding protein alteration. In fact only female rats exhibited decreased CREB activity in the hippocampus (-30%) and prefrontal cortex (-24%) and increased CREB activity in the NAC (+80%) paralleled by increased dynorphin expression (+100%). We also observed altered synaptic markers expression ( $\downarrow$ synaptophysin,  $\downarrow$ PSD95) in the prefrontal cortex of female pretreated rats.

Adolescent exposure to THC did not alter short term memory but reduced spatial memory (radial maze) being males more sensitive than females. According to this result we found significant alteration in pre- and post-synaptic proteins expression ( $\downarrow$ VAMP2,  $\downarrow$ PSD95) in the hippocampus of male pretreated rats.

The present results suggest that cannabis consumption during adolescence may induce long term and gender dependent behavioural effects coupled with stable alteration in neurochemical markers and synaptic plasticity.

**SYNERGISTIC CYTOTOXICITY, INHIBITION OF SIGNAL TRANSDUCTION PATHWAYS AND MODULATION OF GENE EXPRESSION BY SORAFENIB AND GEMCITABINE IN HUMAN PANCREATIC CANCER CELLS****Ricciardi Simona***Divisione di Farmacologia e Chemioterapia, Dipartimento di Medicina Interna, Università degli Studi di Pisa Via Roma 55, 56126 Pisa.**E-mail: simonaricciardi1@yahoo.it*

Pancreatic cancer is one of the most lethal tumours and, although gemcitabine produces a clinical meaningful response, there has been little improvement in prognosis. Therefore, research effort has focused on target-specific agents, such as sorafenib, which blocks both the RAF/MEK/ERK signalling pathways and receptors involved in neovascularization and tumour progression, including VEGFR-2 and c-Kit. We investigated whether sorafenib would be synergistic with gemcitabine against pancreatic cancer cell lines. Cells were treated with sorafenib and gemcitabine, alone or in combination and pharmacologic interaction was studied using the combination index (CI) method. Cell cycle was investigated with flow cytometry. Moreover, the effects of drugs on Akt (S473) and c-Kit (Y823) and ERK (pTpY185/187) phosphorylation, and on apoptosis induction were studied with ELISA and fluorescence microscopy respectively. Finally, quantitative PCR analysis was performed to assess whether sorafenib modulated the expression of the gemcitabine activating enzyme deoxycytidine kinase (dCK) and the drug target ribonucleotide reductase (RR). Sorafenib was cytotoxic against MIA PaCa-2, Capan-1, PANC-1, PANC-2 and BxPc3 cells with IC<sub>50</sub>s of 3.48, 0.61, 4.56, 2.74, and 1.33  $\mu$ M, respectively. A dose dependent inhibition of cell growth was observed after gemcitabine and sorafenib treatment; the CI analysis showed that both schedules of two drugs exhibited synergism in all cell lines. Flow cytometric studies demonstrated that gemcitabine enhanced cellular population in the S phase (from 8.9% in PANC-1 to 14.8% in MIA PaCa-2 cells), whereas sorafenib was not able to significantly modulate cell cycle distribution. Cell exposure to gemcitabine resulted in a significant Akt phosphorylation inhibition, whereas sorafenib exposure reduced c-Kit and ERK phosphorylation in all cell lines. Fluorescence microscopy demonstrated that cells treated with drugs and their combinations presented typical apoptotic morphology; in particular, drug combinations significantly increased ( $P < 0.05$ ) apoptotic index with respect to single agents in Capan-1, MIA PaCa-2 and PANC-1 cells. In PANC-2 and BxPc3 cells the most effective induction of apoptosis was observed after gemcitabine exposure. PCR showed that sorafenib reduced the expression of RRM1 and RRM2 in MIA PaCa-2, Capan-1 and PANC-1 cells, enhancing the dCK/(RRM1xRRM2) ratio ( $P < 0.05$ ).

These data demonstrate that sorafenib and gemcitabine synergistically interact against pancreatic tumour cells, through suppression of Akt, c-Kit and ERK phosphorylation, induction of apoptosis and reduction of RRM1 and RRM2 gene expression, thus providing the experimental basis for developing this combination for the treatment of pancreas cancer.

**WNT SIGNALLING AND NEURODEGENERATION IN ALZHEIMER'S DISEASE: AN IN VIVO STUDY IN THE TgCRND8 MOUSE MODEL****Rosi Maria Cristina***Dip. di Farmacologia Preclinica e Clinica "Mario Aiazzi Mancini", Università degli Studi di Firenze, Viale Pieraccini,6, 50139, Firenze**E-mail: mariacristina.rosi@unifi.it*

Extracellular senile plaques, made of  $\beta$ -amyloid ( $A\beta$ ) peptide deposits, and intraneuronal neurofibrillary tangles (NFTs), made of hyperphosphorylated microtubule-associated protein tau are the main histo-pathological changes seen in the Alzheimer's disease (AD) brain. Impairment of Wnt signalling has been recently proposed as one of the mechanisms underlying AD pathology, potentially linking the two major histopathological hallmarks of the disease. In AD brain, deficiencies in the Wnt pathway have been reported in degenerating neurons as a consequence of the induction of the selective antagonist Dickkopf-1 (DKK-1), which might therefore be a component of the sequence of events leading to neuronal toxicity in response to  $A\beta$ . In the TgCRND8 mouse model of AD, which harbours a double mutant human APP 695 (KM670/671NL+V717F), we investigated: 1) whether and how an abnormal processing of tau takes place; 2) the Wnt signalling pathway by evaluating the expression pattern of DKK-1 and GSK-3 $\beta$ ; 3) the effectiveness of chronic LiCl treatment in ameliorating the AD-like pathology. Four, 7 and 12 month-old TgCRND8 and age-matched, non-Tg wild type mice were used. Western blotting and immunohistochemical techniques were used to reveal proteins of interest. Cognitive impairments were evaluated in the Step-Down and Morris Water Maze tasks. In the neocortex and hippocampus of aged TgCRND8 mice, we detected an abnormal hyperphosphorylation of tau at different sites recognized by PHF-1, AT100, AT8 and CP13 antibodies. Numerous DKK-1 immunopositive neurons were detected in the II, III, V and VI layers of the parietal cortex, in the piriform cortex, in the CA1 and CA3 subfields of the hippocampus and in the Nucleus Basalis Magnocellularis (NBM) of TgCRND8 mice, as compared to control mouse brain. Western blot analysis with DKK-1 antibody confirmed the immunohistochemical data. In the 7-month-old TgCRND8 mouse brain, double-labelling experiments revealed the presence of DKK-1 within phospho-tau-bearing neurons and in the vicinity of amyloid plaques. Moreover, GSK-3 $\alpha$  (Tyr<sup>279</sup>)/ $\beta$  (Tyr<sup>216</sup>) immunoreactivity was increased in the parietal neocortex and in the CA3 area and dentate gyrus of the hippocampus of 12-month-old TgCRND8 mice. To investigate whether GSK-3 $\beta$  inhibition can affect AD pathology *in vivo*, 2-month-old-TgCRND8 (n=8) and control mice (n=8) received i.p. injections of either 0.6 M lithium chloride (10  $\mu$ l per g of body weight) or sterile NaCl (10  $\mu$ l per g of body weight) daily for 30 days. Lithium-treated Tg mice showed a significant improvement ( $P < 0.001$ , Bonferroni post-test) in working memory performance in the Step-Down inhibitory avoidance task with respect to vehicle-treated animals. In the Morris Water Maze task a significant improvement in learning ability ( $P < 0.05$ , Bonferroni post-test) as well as in spatial memory performances ( $P < 0.01$ ) was found in the lithium treated TgCRND8 mice with respect to the vehicle treated Tg controls. In addition, lithium treatment lowered  $A\beta$  burden and decreased GFAP reactivity in TgCRND8 mice.

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## COMPARISON BETWEEN THE CENTRAL ANTINOCICEPTIVE EFFECT OF N-ARACHIDONOYL-PHENOLAMINE (AM-404) AND OF PARACETAMOL: BEHAVIOURAL AND BIOCHEMICAL EVALUATIONS

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N-arachidonoyl-phenolamine (AM404) is the major product of the metabolism of paracetamol (PARA). It has been demonstrated that AM404 is a potent agonist of TRPV<sub>1</sub>, a ligand of CB<sub>1</sub> receptors and an inhibitor of cellular anandamide uptake. The antinociceptive effect produced by cannabinoids seems to be mediated by activation of CB<sub>1</sub> receptors; indeed, the antinociceptive activity of AM404 is antagonized by CB<sub>1</sub> receptor antagonists (1).

The cannabinoid-induced antinociception seems to depend, to some extent, also on the release of opioid peptides into the brain and on the activation of  $\mu$  receptors. We have previously studied the antinociceptive effect of PARA and the modification it produced in the serotonergic system. The antinociceptive effect of PARA matched an increase in serotonin levels and a decrease in the number of 5-HT<sub>2</sub> receptors in the cerebral cortex and in the pons of the rat. Moreover, the PARA-induced antinociception was shown to be partially mediated by the opioidergic system in the brain (2).

The aim of our work was then to compare the AM404 and PARA activity on opioidergic and serotonergic systems, using behavioural and biochemical tests.

We investigated the possible role of the opioidergic system in the antinociceptive effect of AM404 (10 mg/kg i.p.) and PARA (400 mg/kg i.p.) using naloxonazine (a selective  $\mu_1$  receptor antagonist; 10 mg/kg i.p.), 24 h before AM404 or PARA. We assessed the implication of serotonergic system in the AM404 and PARA-induced antinociceptive effect using: a) 5-HT<sub>1A</sub> (NAN-190 5 mg/kg i.p.), 5-HT<sub>2</sub> (ketanserin 5 mg/kg s.c.) and 5-HT<sub>3</sub> (ondansetron 2 mg/kg s.c.) receptor antagonists, injected 15 min before AM404 or PARA; b) evaluating possible changes in 5-HT and 5-HIAA levels in the frontal cortex and in the pons of the rat. Hot-plate test (1 h after the last treatment) was used to evaluate pain threshold and HPLC for biochemical determinations. The antinociceptive effect of AM404 was lesser than that of PARA (%MPE values: CTRL=2.2±1.1; AM404=18.1±4.2; PARA=31.3±6.3, means±S.E.M.; ANOVA followed by Bonferroni test) and was completely prevented by both naloxonazine and ondansetron while PARA was antagonized by naloxonazine and ketanserin. AM404 did not change the 5-HT and 5-HIAA levels in any area studied; PARA significantly increased the 5-HT levels but not 5-HIAA ones, decreasing the turnover ratio.

We observed a different involvement of serotonin receptor subtypes in the antinociceptive activity of the two drugs and a reduction of the serotonergic activity only in the PARA-treated rats. We conclude that the mechanisms of the antinociceptive effect of AM404 and PARA differs in some way, suggesting that this biotransformation does not completely explain the mechanism by which PARA works.

### References:

1. Palazzo E, et al. (2006) Eur J Neurosci 24: 2011-20.
2. Pini LA, Vitale G, Ottani A, Sandrini M (1997) J Pharmacol Exp Ther 280: 934-40.

**ACTIVATION OF PROTEINASE-ACTIVATED RECEPTOR-2 REDUCES AIRWAYS INFLAMMATION IN EXPERIMENTAL ALLERGIC ASTHMA**

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Proteinase-activated receptors (PAR)-2 are members of the family of G-protein coupled receptors activated by proteases. These receptors are widely expressed in several tissues and in virtually all cells involved in rhinitis and asthma. In particular, proteinases activating PAR-2 may affect airway functions and play a role in human diseases.

Aim of this study was: 1) to assess the role of PAR-2 in bronchoconstriction, airway responsiveness, and immune response after allergic challenge, in rabbits sensitized to Par j1, the major allergen of *Parietaria judaica* pollen; to evaluate antigen challenge in rabbits treated with PAR-2 activating peptide (PAR-2AP) (SLIGRL) or the scrambled peptide LSIGRL or vehicle immediately before allergen exposure measuring airway responsiveness; 3) to characterize bronchoalveolar lavage following histamine challenge and phenotype analysis of cells by flow cytometry and analysis of cytokine production by quantitative PCR.

PAR-2AP pre-treatment, but not the scrambled peptide, was able to significantly inhibit bronchoconstriction, airway hyperresponsiveness, and to modulate the immune response induced by allergic challenge in sensitized rabbits. Western blot analysis showed a clear up-regulation of PAR-2 in rabbit lungs challenged with Par j1. The phenotype analysis of the cells recovered from BAL ( bronchoalveolar lavage) showed an increase in RLA-DR (rabbit Class II antigen) positive cells while RTLA (rabbit T Cell lymphocyte antigen) positive cells were unchanged. IFN- $\gamma$  ( interferon  $\gamma$ ) and IL-2 ( interleuchin- 2) production were inhibited with a concomitant increase in IL-10 of about 10 fold over the control values.

In this experimental model, PAR-2 modulates bronchoconstriction interfering with antigen challenge-induced immune response, in rabbits sensitized and challenged to Par j1.

## ADENOSINE MODULATES VASCULAR ENDOTHELIAL GROWTH FACTOR EXPRESSION VIA HYPOXIA-INDUCIBLE FACTOR-1 IN HUMAN GLIOBLASTOMA CELLS

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Hypoxia appears to induce a program that shifts the cellular phenotype toward an increase in extracellular adenosine. Hypoxia-inducible factor-1 (HIF-1) is a key regulator of genes crucial to many aspects of cancer biology. Since in gliomas there is a strong correlation between HIF-1 $\alpha$  expression, tumour grade and tumour vascularisation, the aim of this study was to investigate whether adenosine may regulate HIF-1 in human glioblastoma cell lines.

For hypoxic conditions, U87MG and A172 GBM cells were placed for the indicated times in a modular incubator chamber and flushed with a gas mixture containing 1% O<sub>2</sub>, 5% CO<sub>2</sub> and balance N<sub>2</sub>.

We performed experiments with different methods such as: Western blot analysis to investigate the expression of HIF-1 $\alpha$  protein; small interfering RNA (siRNA) design; RT-PCR experiments to quantify human HIF-1 $\alpha$ , vascular endothelial growth factor (VEGF), and A<sub>3</sub> mRNA transcripts. The levels of VEGF protein secreted by the cells in the medium were determined by a VEGF ELISA kit and finally the cells were prepared for the luciferase reporter assay.

The results indicate that in the human hypoxic A172 and U87MG glioblastoma cell lines adenosine up-regulates HIF-1 $\alpha$  protein expression via the A<sub>3</sub> receptor subtype. In particular, we investigated the effect of A<sub>3</sub> receptor antagonists on HIF-1 and VEGF expression. We found that A<sub>3</sub> antagonists inhibit adenosine-induced HIF-1 $\alpha$  and VEGF protein accumulation in the hypoxic cells. Investigations in the molecular mechanism showed that A<sub>3</sub> receptor stimulation activates p44/p42 and p38 MAPKs that are required for A<sub>3</sub>-induced increase of HIF-1 $\alpha$  and VEGF.

The data presented in this study provide the indications that: adenosine can further potentiate the effect of hypoxia on HIF-1 $\alpha$  and VEGF expression in human GBM cells; both are increased via A<sub>3</sub> receptor stimulation; MEK and p38 MAPK may have a role in A<sub>3</sub> receptor ability to improve HIF-1 $\alpha$  and VEGF protein expression.

**TIMING OF ESTROGEN REINTRODUCTION AFTER OVARIECTOMY AFFECTS EX VIVO VASCULAR REACTIVITY IN THE RAT AORTA****Sanvito Paola***Dipartimento di Farmacologia ed Anestesiologia "E. Meneghetti", Università degli Studi di Padova, Largo Meneghetti 2, 35131 Padova**E-mail: paola.sanvito@unipd.it*

The physiologic hormone 17 $\beta$ -estradiol (E<sub>2</sub>) has direct protective effects on the cardiovascular system (1). One of its most important actions in the vessel wall is estrogen receptor  $\alpha$  (ER $\alpha$ )-mediated vasodilatation, which occurs through both genomic and nongenomic activation of endothelial nitric oxide synthase (eNOS) (2-3). Yet evidence from basic research on beneficial cardiovascular effects of estrogen has not been effectively translated into clinical practice. To test if early estrogen therapy soon after ovarian hormone deprivation is critical to vascular function, we implanted ovariectomized (OVX) rats with E<sub>2</sub> or oil capsules 1, 4, and 8 months after surgery. Cumulative concentration-response curves to acetylcholine (1 nM-10  $\mu$ M), E<sub>2</sub>, and the ER $\alpha$  selective agonist PPT (0.1 pM-100 nM) were obtained in aortic preparations precontracted with noradrenaline (EC<sub>60</sub>). Two aortic rings from 3 to 5 rats were used in each set of experiments. ER $\alpha$  mRNA levels, eNOS, and phosphorylated eNOS protein expression were evaluated in aortic endothelial lysates obtained from each experimental group. The relaxant response of isolated aortic rings to acetylcholine was attenuated 8 months after estrogen deprivation (E<sub>max</sub>=73.4 $\pm$ 0.1%; P<0.05 vs. 1-month OVX) and only partially restored by *in vivo* estrogen administration (E<sub>max</sub>=81.4 $\pm$ 0.4%). The rapid vasorelaxing responses to E<sub>2</sub> and to the ER $\alpha$  selective agonist PPT, previously shown in aortas from intact female rats (3), waned at any time post-ovariectomy and were restored by E<sub>2</sub> treatment at 1 and 4 months (E<sub>max</sub>=25.6 $\pm$ 2.6% and E<sub>max</sub>=21.9 $\pm$ 1.2%, respectively), but not 8 months post-ovariectomy (E<sub>max</sub>=8.4 $\pm$ 0.4%; P<0.05 vs. 1-month OVX). This was associated with a 3-fold decline in ER $\alpha$  mRNA expression in the aortic endothelium from 8-month vs. 1-month OVX animals as measured by RT-PCR. Whereas the levels of eNOS protein were unchanged by hypoestrogenicity or E<sub>2</sub> treatment at all time points, the amount of active phosphorylated eNOS significantly rose following E<sub>2</sub> treatment after 1 and 4 months, but not 8 months post-ovariectomy. The present findings document that the functional impairment of the ER $\alpha$ /eNOS signalling network after an extended period of hypoestrogenicity was insensitive to estrogen replacement, thus providing experimental support to early initiation of estrogen replacement with preferential ER $\alpha$  drugs to improve cardiovascular outcomes.

## References:

1. Mendelsohn ME, Karas RH (2005) *Science* 308: 1583-7.
2. Chen Z, et al. (1999) *J Clin Invest* 103: 401-6.
3. Bolego C, et al. (2005) *J Pharmacol Exp Ther* 313: 1203-8.

## IN VITRO EFFECTS OF SULFORAPHANE ON CELL VIABILITY, APOPTOSIS INDUCTION AND CELL-CYCLE MODULATION IN HYPOXIA CONDITIONS

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The association of decreased cancer risk with intake of cruciferous vegetables is stronger than that reported for fruit and vegetables in general. An active constituent in cruciferae is sulforaphane (SUL). *In vitro* and *in vivo* studies have reported that SF affects many steps of cancer development. It can modulate early stages of carcinogenesis process or affect events, such as apoptosis, cell proliferation and angiogenesis more specifically involved in the promotion and progression phases. SN is a potent proapoptotic agents in a wide variety of cancer cells both *in vitro* and *in vivo*, including acute lymphoblastic leukaemia cell lines. Hypoxia is a defined characteristic of solid tumours. However, recent studies have shown that hypoxic conditions can be recognized also in non-solid tumours, such as in the leukaemia bone marrow, where the blasts responsible for this pathology are located (1-3). The responses to low O<sub>2</sub> levels contribute substantially to the malignant phenotype and chemoresistance. In this study the effects on cell viability, cell cycle and apoptosis of SF in hypoxic conditions were evaluated and compared to those observed in normoxic conditions.

Jurkat cells (T-lymphoblastoid phenotype) was cultured in hypoxic (1.5% O<sub>2</sub>) conditions. Analysis of cell-cycle, and apoptosis induction was performed using flow cytometry.

The results obtained show that SUL induces cytotoxic effects on Jurkat cells independently of the culture conditions used, even if, the effects are more pronounced in normoxic conditions. Flow cytometric assessment of apoptosis revealed that SUL is able to induce apoptotic death in both normoxic and hypoxic conditions. Although its effect on apoptosis induction is lower in hypoxic conditions, in this case the induction seems to appear at earlier time of treatment if compared with normoxic conditions. Interestingly, SUL has also strong anti-proliferative effect. In normoxic conditions SUL blocks the cell-cycle progression in G2/M phase while in hypoxic conditions this block occurs in G1 phase. In addition, the intensity of the block appears to be more marked in hypoxic conditions. Taken together, our results underline an *in vitro* anti-tumour effect of SUL in hypoxia conditions. Moreover, the differences observed lead us to continue the studies in order to uncover the molecular mechanisms underlying this behaviour.

### References:

1. Mortensen BT, et al. (1998) Br J Haematol 102: 458-64.
2. Semenza GL (2002). Trends Mol Med 8: S62-7.
3. Semenza GL (2000) Crit Rev Biochem Mol Biol 35: 71-103.

**CAFFEINE INHIBITS ADENOSINE-INDUCED ACCUMULATION OF HYPOXIA-INDUCIBLE FACTOR-1 $\alpha$ , VASCULAR ENDOTHELIAL GROWTH FACTOR, AND INTERLEUKIN-8 EXPRESSION IN HYPOXIC HUMAN COLON CANCER CELLS**

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Frequent coffee consumption has been associated with a reduced risk of colorectal cancer in a number of case-control studies. Coffee is a leading source of methylxantines, such as caffeine. The induction of vascular endothelial growth factor (VEGF) and interleukin-8 (IL-8) is an essential feature of tumour angiogenesis, and the hypoxia-inducible factor-1 (HIF-1) transcription factor is known to be a key regulator of this process.

In this study, we investigated the effects of caffeine on HIF-1 protein accumulation and on VEGF and IL-8 expression in the human colon cancer cell line HT29 under hypoxic conditions. HT29 human tumour colon cells were obtained from American Tissue Culture Collection (ATCC). Human umbilical vein endothelial cells (HUVEC), tissue culture media and growth supplements were obtained from Cambrex (Bergamo, Italy). We performed our experiments with different methods such as binding, measurement of cAMP levels, JAM test to measure cell death, MTS assay to determine colon cell viability and proliferation, migration assay, densitometry analysis, treatment of cells with siRNA, ELISA, and Western blot analysis. Data sets were examined by analysis of variance (ANOVA) and Dunnett's test (when required). A P value <0.05 was considered statistically significant.

Our results show that caffeine significantly inhibits adenosine-induced HIF-1 $\alpha$  protein accumulation in cancer cells. We show that HIF-1 $\alpha$  and VEGF were increased through A3 adenosine receptor stimulation, while the effects on IL-8 were mediated via the A2B subtype. Pretreatment of cells with caffeine significantly reduced adenosine-induced VEGF promoter-activity and VEGF and IL-8 expression. The mechanism of caffeine seems to involve the inhibition of the extracellular signal-regulated kinase 1/2 (ERK1/2), p38 and Akt, leading to a marked decrease in adenosine-induced HIF-1 $\alpha$  accumulation, VEGF transcriptional activation, and VEGF and IL-8 protein accumulation. Functionally, we observed that caffeine also significantly inhibits the A3 receptor-stimulated cell migration of colon cancer cells. Conditioned media prepared from colon cells treated with an adenosine analogue, increased HUVEC migration.

These data provide evidence that adenosine could modulate the migration of colon cancer cells by a HIF-1 $\alpha$ /VEGF/IL-8-dependent mechanism and that caffeine has the potential to inhibit colon cancer cell growth

## CANNABINOID SYSTEM IS IMPLICATED IN PROLIFERATION OF CEREBELLAR NEURAL PROGENITOR CELLS

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The recent discovery of neural progenitor/stem cells in the adult brain has provided strong support for the existence of neurogenesis (i.e., generation of new neurons). The cannabinoid system has been recently shown to promote neurogenesis in the adult hippocampus (1), although the intracellular molecular mechanisms underlying this effect have not been fully understood. Cannabinoids and endocannabinoids exert their actions in the brain through activation of two types of metabotropic receptors, CB1 and CB2, and affect a large number of physiological processes, including learning, memory, emotion, pain perception, immune and inflammatory responses, cardiovascular function, and reproduction.

To elucidate the cellular and molecular mechanisms underlying cannabinoid neurogenic action, we used neural progenitor cells isolated from post-natal cerebellum, as an *in vitro* model of neural cell proliferation. Phenotypical and genotypical characterization of these cells by immunocytochemistry and RT-PCR, respectively, has shown that they were immunoreactive for several immature neuronal markers and possess both cannabinoid receptors, CB1 and CB2. Furthermore, isolated cerebellar progenitor cells could be induced to differentiate into mature neuronal and glial cells by withdrawing the mitogen growth factors. Twenty-four-h treatment of cerebellar neural progenitor cells for 10 days *in vitro* with increasing concentrations (1-1000 nM) of the non-selective synthetic cannabinoid agonists CP-55,940 and WIN-55,212-2 increased cell proliferation, evaluated as [<sup>3</sup>H]thymidine incorporation, by 20.2±7.9% and 35.3±9.5%, respectively. The proliferative response induced by either CP-55,940 or WIN-55,212-2 was completely abolished by the selective CB1 receptor antagonist AM251 (0.1-1 µM), thus suggesting an involvement of CB1 receptor activation. In addition, preliminary results showed that both cannabinoid agonists increased mRNA levels for cyclin D1, which is known to play a critical role in the progression of cell cycle. Research is in progress to define the intracellular transduction pathways leading from CB1 receptor activation to the increase in cyclin D1 expression.

### References:

1. Jiang W, et al. (2005) J Clin Invest 115: 3104-16.
2. Zusso M, Debetto P, Guidolin D, Giusti P (2004) Crit Rev Neurobiol 16: 59-65.

## ROLE OF NO/Ca<sup>2+</sup> SIGNALLING IN MEDIATING CYTOKINE-INDUCED NEUROINFLAMMATION

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Reactive astrogliosis is a neuroinflammatory process present in many neurodegenerative diseases such as multiple sclerosis and Alzheimer's disease, which are generally characterised by cell proliferation and increased expression of glial fibrillary acidic protein. Most pro-inflammatory cytokines, in particular interleukin-1 $\beta$  (IL-1 $\beta$ ) has convincingly implicated in generation or modulation of astrogliosis (1-2), but the specific signalling mechanism by which this cytokine regulates this process and in particular cell proliferation, is still unclear. To this aim, we investigated the effect of IL-1 $\beta$  on the regulation of mitogen activated protein kinases/extracellular signal-regulated kinases (MAPK/ERK), which play a central role in cell growth, and the mechanism underlying this effect in astrocytoma U373-MG cells.

Data showed that, depending on concentration, IL-1 $\beta$  induced a proliferative or an anti-proliferative effect, which paralleled respectively the up- or down-regulation of ERK. The unspecific or the selective iNOS inhibitor, N- $\omega$ -nitro-L-arginine methyl ester (L-NAME) and N-[[3-(aminomethyl)phenyl]methyl]-ethanimidamide dihydrochloride (1400W), respectively, as well as the guanylyl cyclase inhibitor 1H(1,2,4) oxadiazole (4,3-a) quinoxalin 1-one (ODQ), or the ryanodine-plus the IP3-sensitive receptor inhibitor 2-aminoethoxydiphenylborane (2APB), antagonized ERK activation as well as the mitogenic effect of IL-1 $\beta$ . Furthermore, cells treated with IL-1 $\beta$ , showed increased levels of NO and intracellular Ca<sup>2+</sup>.

Treatment of rat striatal slices with IL-6 or TNF- $\alpha$  elicited a dose-dependent elevation of spontaneous <sup>45</sup>Ca<sup>2+</sup> release from tissue, and this effect was counteracted by L-NAME, ODQ, or ryanodine plus 2-APB. On the contrary, the anti-inflammatory cytokine IL-4 failed to elicit the <sup>45</sup>Ca<sup>2+</sup> response. Taken together these data demonstrated that the intracellular NO/Ca<sup>2+</sup> signalling mediated the mitogenic effect of IL-1 $\beta$  via activation of ERK pathway. Furthermore they indicate that the same signalling could represent a more general mechanism mediating the responses of pro-inflammatory cytokines. Therefore modulation of NO/Ca<sup>2+</sup> signalling may represent a plausible strategy for therapeutic intervention to reduce the inflammatory response that characterizes neurodegenerative diseases.

### References:

1. Saura J, et al. (2003) J Neurochem 85: 651-61.
2. Depino A, Ferrari C, Pott Godoy MC, Tarelli R, Pitossi FJ (2005) J Neuroimmunol 168: 96-110.



**ROLE OF TGF-BETA ON MAP KINASE PHOSPHORYLATION AND CELL PROLIFERATION IN PRIMARY CULTURES OF HUMAN LUNG FIBROBLAST**

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Transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) is crucially involved in the fibrotic events characterizing interstitial lung diseases, as well as in the airway remodelling process typical of asthma.

Within such a context, the aim of our study was to investigate, in primary cultures of normal human lung fibroblasts, the effects of TGF- $\beta$ 1 on mitogen-activated protein kinase (MAPK) phosphorylation, cell proliferation and production of interleukins 6 (IL-6) and 11 (IL-11), in the presence or absence of a pretreatment with budesonide.

MAPK phosphorylation was detected by Western blotting, cell count was performed using Trypan blue staining, and the release of IL-6 and IL-11 into cell culture supernatants was assessed by ELISA. TGF- $\beta$ 1 (10 ng/ml) significantly stimulated MAPK phosphorylation ( $P < 0.01$ ), and also enhanced cell numbers as well as the secretion of both IL-6 and IL-11, which reached the highest increases at 72 h of cell exposure to this growth factor. All such effects were prevented by budesonide (10 nM) and, with the exception of IL-6 release, also by a mixture of MAPK inhibitors.

Therefore, our findings suggest that the fibrotic action exerted by TGF- $\beta$ 1 in the lung is mediated at least in part by MAPK activation and by an increased synthesis of the profibrogenic cytokines IL-6 and IL-11; all these effects appear to be prevented by corticosteroids via inhibition of MAPK phosphorylation.

**ROLE OF PROSTAGLANDIN E-2 (PGE-2) ON VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) EXPRESSION AND TUMOUR ANGIOGENESIS IN HUMAN COLON CANCER CELLS**

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Colorectal cancer is one of the leading causes of morbidity and mortality in the Western world. Epidemiological studies suggest that in recent years the number and mortality rate of patients with colorectal cancer increased (1). Recently, it has been reported by our laboratory and others that prostaglandin E-2 (PGE-2) is able to promote tumour cell growth and invasion (2). Tumour angiogenesis promoted by different stimuli as vascular endothelial growth factor (VEGF) is a fundamental step of tumour growth and progression.

The aim of this study was: 1) to investigate in human colon cancer cells the effects of PGE-2 on VEGF expression and tumour angiogenesis; 2) to set up a model of co-culture between tumour and endothelial cells.

The human colon cancer adenocarcinoma cell line HT-29 was used as a model. Cell proliferation induced by PGE-2 was assayed by MTT test. Tumour angiogenesis was evaluated by co-culturing microvascular endothelial cells (CVEC) and HT-29 cells. The production and the expression of the angiogenic growth factor VEGF were evaluated by ELISA and Western blotting, respectively.

PGE-2 at 1  $\mu$ M concentration induced tumour cell to increase their growth and increased VEGF production. Co-culture of CVEC and HT-29 were set up on the effect of the tumour cell on the endothelial cells assayed. Treatment of the tumour cells with PGE-2 resulted in increased angiogenic phenotype of the endothelium.

Our studies indicate that PGE-2 actively contributes to increase the progression of colon cancer cells by augmenting VEGF output and angiogenesis.

References:

1. Dove-Edwin I, Thomas HJ (2001) *Aliment Pharmacol Ther* 15: 323-36.
2. Donnini S, et al. (2007) *FASEB J* 21: 2418-30.

## ATLASTIN IS A NOVEL ENDOPLASMIC RETICULUM PROTEIN INVOLVED IN GOLGI-ENDOPLASMIC RETICULUM TRANSPORT

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The hereditary spastic paraplegias encompass a diverse spectrum of disorders that are characterized by progressive spastic weakness of the lower extremities. The SPG3A gene, encoding the novel protein atlastin, has been identified as the locus responsible for a form of paraplegia characterized by the earliest onset. Atlastin protein sequence shows homology to large GTPases of the dynamin superfamily. The function of atlastin is unknown, and consequently the pathological mechanism underlying disease remains unknown.

We have identified and cloned the fly homologue (D-atlastin) of human Atlastin and raised an antibody against the fly protein. We carried out a detailed analysis of the embryonic and larval expression patterns as well as the subcellular localization of D-atlastin. D-atlastin is ubiquitously expressed during all stages of *Drosophila* development. At the subcellular level D-atlastin is highly enriched in and co-localizes with endoplasmic reticulum (ER) markers. A small amount of signal is also detected in the Golgi apparatus suggesting an involvement of this protein in vesicular trafficking between ER and Golgi.

To clarify the biological role of D-atlastin we generated transgenic flies for its overexpression and knockdown *in vivo*. Ubiquitous overexpression of D-atlastin causes death around stage 13 of embryonic development. Analysis of tissues overexpressing D-atlastin showed morphological alterations of the Golgi, which disassembles and colocalizes with markers for ER. In contrast to overexpression, loss of D-atlastin allowed survival of few escapers with most individuals dying at pupal stage. Eclosed adults had a short lifespan and an obvious reduction of size.

Our results show that atlastin is important for vesicular transport between ER and Golgi and necessary for proper development.

### References:

1. Zhu PP, et al. (2003) *J Biol Chem* 278: 49063-71.
2. Zhu PP, et al. (2006) *Hum Mol Genet* 15: 1343-53.
3. Lee Y. et al. (2006) *Neurobiol Aging*. Oct 6
4. Namekawa M, et al. (2007) *Mol Cell Neurosci* 35: 1-13.

## PHARMACOLOGICAL CHARACTERIZATION OF ADENOSINE RECEPTORS IN BOVINE CHONDROCYTES

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Adenosine mediates a number of physiological functions through the interaction with 4 cell surface receptors named  $A_1$ ,  $A_{2A}$ ,  $A_{2B}$ , and  $A_3$ .

Chronic inflammation is a significant factor in the pathophysiology of many forms of joint diseases. Adenosine has been reported to reduce inflammation in several *in vivo* models, suggesting a potential value of adenosine as therapeutic mediator of inflammatory joint disease able to limit articular cartilage. To date in chondrocytes adenosine binding parameters have not been investigated even if it is known that these cells respond to extracellular adenosine. The present study was designed to evaluate the binding and functional characterization of  $A_1$ ,  $A_{2A}$ ,  $A_{2B}$ , and  $A_3$  adenosine receptors in bovine chondrocytes membranes.

Chondrocytes were isolated from cartilage fragments obtained from the weight-bearing region of the bovine articular cartilage. Saturation binding experiments were performed using high affinity radioligands: [ $^3$ H]DPCPX, [ $^3$ H]ZM241385, [ $^3$ H]MRE2029F20, and [ $^3$ H]MRE3008F20 for the adenosine receptors  $A_1$ ,  $A_{2A}$ ,  $A_{2B}$ , and  $A_3$  respectively. In the cAMP assay the potency of typical high affinity  $A_{2A}$  or  $A_3$  agonists was evaluated. Thermodynamic analysis was studied to investigate the forces driving drug-receptor coupling.

Chondrocytes expressed all the adenosine receptors. The affinity ( $K_d$ ) and the receptor density ( $B_{max}$ ) of the  $A_1$  receptors were  $2.18 \pm 0.22$  nM and  $41 \pm 2$  fmol/mg of protein, respectively.  $A_{2A}$  receptors showed  $K_d = 1.71 \pm 0.32$  nM and  $B_{max} = 53 \pm 5$  fmol/mg of protein.  $A_{2B}$  receptors revealed a  $K_d$  of  $2.19 \pm 0.24$  nM and  $B_{max}$  of  $53 \pm 3$  fmol/mg of protein.  $A_3$  receptors binding parameters were  $4.61 \pm 0.35$  nM and  $79 \pm 5$  fmol/mg of protein, respectively. Thermodynamic parameters indicated that the binding to adenosine receptors is enthalpy- and entropy-driven. The affinity ( $K_i$ ) of CGS 21680 and Cl-IB-MECA were  $28 \pm 3$  nM and  $2.6 \pm 0.3$  nM, respectively. The potency ( $EC_{50}$ ) of CGS 21680 was  $82 \pm 7$  nM and the  $IC_{50}$  of Cl-IB-MECA was  $6.33 \pm 0.64$  nM.

This study shows for the first time the presence of adenosine receptors in bovine chondrocytes suggesting that their modulation could be used for pharmacological interventions in inflammatory joint diseases.

**ROLE OF ADENOSINE A<sub>3</sub> RECEPTORS ON CA1 HIPPOCAMPAL NEUROTRANSMISSION DURING OXYGEN-GLUCOSE DEPRIVATION EPISODES OF DIFFERENT DURATION****Traini Chiara**

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The role of adenosine A<sub>3</sub> receptors in synaptic transmission under severe (7 min) and shorter (2-5 min) ischemic conditions, obtained by oxygen and glucose deprivation (OGD), was investigated in rat hippocampal slices.

The effects of selective A<sub>3</sub> agonists or antagonists were examined on field excitatory postsynaptic potentials (fEPSPs) extracellularly recorded at the dendritic level of the CA1 pyramidal region. In all slices, 7 min OGD evoked tissue anoxic depolarization (AD, peak at approximately 7 min from OGD start, n=13), a phenomenon strictly correlated with the extent of brain damage during ischemia both *in vivo* and *in vitro* (1). In addition, 7 min OGD were invariably followed by irreversible loss of electrically evoked fEPSPs (n=13). Brief periods of OGD (2 or 5 min) were followed by complete recovery of neurotransmission and imply shorter times of A<sub>3</sub> receptor stimulation by endogenous adenosine released during the ischemic episode.

The novel, selective A<sub>3</sub> antagonist LJ1251 [(2*R*,3*R*,4*S*)-2-(2-chloro-6-(3-iodobenzylamino)-9*H*-purin-9-yl)tetrahydrothiophene-3,4-diol, 0.1-10 nM] protected hippocampal slices from irreversible fEPSP depression induced by 7 min OGD and prevented or delayed the appearance of AD, without affecting fEPSPs in normoxic conditions.

Similar results were obtained when severe OGD was carried out with a long, receptor-desensitizing exposure to various selective A<sub>3</sub> agonists: 5'-N-methylcarboxamidoadenosine derivatives Cl-IB-MECA [N<sup>6</sup>-(3-iodobenzyl)-2-chloro, 10 nM, n=6], VT72 [N<sup>6</sup>-methoxy-2-phenylethynyl, 10 nM, n=6], VT158 [N<sup>6</sup>-methoxy-2-phenylethynyl, 5 nM, n=3], VT160 [N<sup>6</sup>-methoxy-2-(2-pyridinyl)-ethynyl, 5 nM, n=4], VT163 [N<sup>6</sup>-methoxy-2-*p*-acetylphenylethynyl, 5 nM, n=3], and AR132 [N<sup>6</sup>-methyl-2-phenylethynyladenosine, 10 nM, n=5].

The selective A<sub>3</sub> antagonist MRS1523 (3-propyl-6-ethyl-5-[(ethylthio)carbonyl]-2-phenyl-4-propyl-3-pyridine carboxylate, 100 nM) reduced fEPSP depression evoked by 2-min OGD and induced a faster recovery of fEPSP amplitude after 5-min OGD. Similar results were obtained for 2- or 5-min OGD applied in the presence of each of the A<sub>3</sub> agonists tested. Shorter exposure to A<sub>3</sub> agonists (only 2 min) significantly delayed the recovery of fEPSP amplitude after 5-min OGD.

Our results indicate that A<sub>3</sub> receptors, stimulated by selective A<sub>3</sub> agonists, undergo desensitization during OGD. It is inferred that CA1 hippocampal A<sub>3</sub> receptors stimulated by adenosine released during brief ischemia (2 and 5 min) exert A<sub>1</sub>-like protective effects on neurotransmission. Severe ischemia would transform the A<sub>3</sub> receptor-mediated effects from protective to injurious.

Increased knowledge of molecular mechanisms of A<sub>3</sub> receptors during cerebral ischemia may increase our understanding of the utility of selective A<sub>3</sub> agonists/antagonists for treatment of ischemic and other neurodegenerative disorders.

## References:

1. Somjen GG (2001) *Physiol Rev* 81: 1065-96.

**ENDOCANNABINOID AND PURINERGIC SYSTEMS IN RODENT CHRONIC CONSTRICTION INJURY: A NEUROPATHIC PAIN MODEL****Trovato Anna Elisa***Dipartimento di Farmacologia, Chemioterapia e Tossicologia Medica, Università degli Studi di Milano, via Vanvitelli 32, 20129 Milano**E-mail: anna.trovato@unimi.it*

Neuropathic pain is a personally devastating and costly condition affecting 3-8% of population. Existing pharmacological treatments have limited effectiveness and produce frequent adverse effects. The role of both endocannabinoid and purinergic systems in pain is well known. The discovery of the endocannabinoid system [cannabinoid receptors (CBR), endogenous cannabinoids, anandamide (AEA) and 2-arachidonylglycerol, endocannabinoid biosynthesis and inactivation systems] has greatly expanded the opportunity for examining the therapeutic effect of its modulation in neuropathic pain. This study tested whether direct or indirect activation of CBR or AEA transport inhibition could relieve the neuropathic nociceptive hypersensitivity. Mononeuropathy was induced in male Wistar rats by the sciatic nerve chronic constriction (CCI). Neuropathic rats were administered with WIN55,212-2, a synthetic cannabinoid, or AM404, AEA uptake inhibitor, starting from the day after injury, or with cannabidiol, a non-psychoactive component of cannabis, from the day 7, when painful symptoms were well evident. All compounds were able to abolish in a time- and dose-dependent manner both hyperalgesia and allodynia (measured by Plantar test, Randall-Selitto test, and Dynamic Plantar Aesthesiometer) in the injured hind limb. CBR and transient receptor potential vanilloid 1 (TRPV1: transient receptor potential vanilloid 1) are up regulated during neuropathy, making the cannabinoid system very sensitive. The co-administration of antagonists specific for CBR and TRPV1 demonstrated the direct or indirect involvement of all these receptors in anti-nociceptive activity of these drugs. The anti-hyperalgesic and anti-allodynic effect of these endocannabinoid-system modulators is mediated not only by CB and TRPV1 receptors, but also by direct or indirect action on different pro-inflammatory and nociceptive systems, such as nitric oxide/nitric oxide synthase (NO/NOS), pro-inflammatory cytokines, cyclooxygenase, oxidative stress and the nuclear transcription factor NF- $\kappa$ B, which mediates trans-activation of genes encoding for these proteins (fluorimetric, Western blot, spectrophotometric and ELISA procedures were employed) (1-3).

Neurotransmitter ATP, that activates purinoceptors, appears to play a role in pain development and transmission. Therefore, the second aim of this study was to evaluate the possible effects of purinergic antagonists on neuropathic pain behaviour. Pyridoxal-phosphate-6-azophenyl-2',4'-disulfonic acid (PPADS), a non-selective purinoceptor antagonist, administered once a day for 11 days, from day 3 after the sciatic nerve injury, in male mice C57BL/6J, attenuated mechanical allodynia and hyperalgesia in a time- and dose-related manner. Simultaneously, PPADS reversed the iNOS and nNOS nitric oxide/nitric oxide synthase over-production, the pro-inflammatory cytokine over-expression (real time RT-PCR) and NF- $\kappa$ B over-activation in peripheral and central nervous system steps involved in pain development and transmission (4). The PPADS: pyridoxal-phosphate-6-azophenyl-2',4'-disulfonic acid treatment has a therapeutic efficacy, since the nociceptive hypersensitivity did not appear for 20 days after the treatment interruption. PPADS precociously reversed the decreased P0 myelin protein mRNA (RNAase protection assay) caused by injury, and ameliorated the sciatic nerve morphology (histological analysis). The administration of antagonists specific for the purinoceptor subtypes, more involved in neuropathic pain, such as isoPPADS for P2X<sub>1</sub>, oxATP for P2X<sub>7</sub> and A317491 for P2X<sub>3-2/3</sub>, showed that neuronal P2X<sub>3-2/3</sub> play a pivotal role in neuropathic nociceptive hypersensitivity.

In conclusion, both systems are activated following neuropathy, particularly in the pain-involved areas. They operate in two different manners: the cannabinoid system regulates the pain symptomatology by inhibiting it; while the over-released ATP after injury increases the pain sensations. Consequently, the endocannabinoid system stimulation on one hand and the reversal of purinoceptor over-activation on the other can be valid alternative strategies for neuropathic pain relief.

## References:

1. Costa B, et al. (2004) Br J Pharmacol 141: 4-8.
2. Costa B, et al. (2006) Br J Pharmacol 148: 1022-32.
3. Costa B, et al. (2007) Eur J Pharmacol 556: 75-83.
4. Trovato AE, et al. (2007) Pain (in press).

## THE NK1 RECEPTOR ANTAGONIST SSR140333 AMELIORATES EXPERIMENTAL COLITIS IN RATS

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Inflammatory bowel disease is characterized by increased visceral perception and nociception. Several lines of evidence showed that inflammation is associated with changes in the expression of tachykinins both in human and animal models. Tachykinins, including substance P (SP), are small peptides expressed in the extrinsic primary afferent nerve fibres and enteric neurons of the gut: they exert their action through 3 distinct receptors, termed NK<sub>1</sub>, NK<sub>2</sub>, and NK<sub>3</sub>. SP modulates intestinal motility and enteric secretion, acting preferentially through the NK<sub>1</sub> receptor (1-2). SP neural network and NK<sub>1</sub> receptor expression are increased in patients with inflammatory bowel disease, and similar changes were observed in experimental models of inflammation (3). The 2,4 dinitrobenzene sulphonic acid (DNBS) model of colitis is useful to study innate immunity, non-specific inflammation, and wound healing (4); it has been suggested that the transmural inflammation seen in this model resembles that found in Crohn's disease and can therefore be used to study which cells and mediators are involved in this type of inflammation.

Aim of this study was to test the possible protective effect of the NK<sub>1</sub> receptor antagonist SSR140333 on: 1) acute model of intestinal inflammation; 2) reactivation of DNBS-induced colitis in rats.

Acute colitis was induced in male Sprague-Dawley rats by intrarectal administration of DNBS (15 mg/rat in 50% ethanol). Reactivation of colitis was induced by intrarectal injections of DNBS on day 28 (7.5 mg/rat in 35% ethanol). Animals were sacrificed on day 6 (acute colitis) and 29 (reactivation of colitis). SSR140333 (10 mg/kg) was administered orally starting from the day before the induction of colitis for 7 days (acute colitis) or seven days before the reactivation of colitis. Colonic damage was assessed by means of macroscopic and microscopic scores and myeloperoxidase (MPO) activity. Statistical analysis was performed using ANOVA (one-way or two-way, as appropriate) with the Bonferroni's correction for multiple comparisons.

DNBS administration impaired body weight gain and markedly increased all inflammatory parameters ( $P < 0.01$ ). Treatment with SSR140333 10 mg/kg significantly counteracted the impairment in body weight gain, decreased macroscopic and histological scores and reduced colonic MPO activity ( $P < 0.01$ ) (acute model). Similar results were obtained administering the SSR140333 (3 and 10 mg/kg) for 5 days, starting the day after the induction of colitis. Intrarectal administration of DNBS 4 weeks after the first DNBS administration resulted in reactivation of colitis, with increases in macroscopic and histological damage scores and increase in MPO activity. Preventive treatment with SSR140333 10 mg/kg decreased macroscopic damage score, significantly reduced microscopic damage score but did not affect MPO activity.

Treatment with SSR140333 reduced significantly intestinal damage in acute model of intestinal inflammation in rats. The NK<sub>1</sub> receptor antagonist SSR140333 was also able to prevent relapse in experimental colitis. These results support the hypothesis of SP involvement in intestinal inflammation and indicate that NK receptor antagonists may have a therapeutic potential in inflammatory bowel disease.

### References:

1. Evangelista S (2001) *Curr Pharm Des* 7: 19-30.
2. Rijniere A, van Zijl KM, Koster AS, Nijkamp FP, Kraneveld AD (2006) *Eur J Pharmacol* 548: 150-7.
3. Mantyh CR, et al. (1995) *Gastroenterology* 109: 850-60.
4. Elson CO, Sartor RB, Tennyson GS, Riddell RH (1995) *Gastroenterology* 109: 1344-67.

**EFFECTS OF ESTROGENS ON RODENTS URINARY BLADDER: GENDER DIFFERENCES IN ANOXIA-GLUCOPENIA DAMAGE****Valeri Aurora**

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Some bladder disorders, such as obstructive bladder dysfunction and hyperactivity, may be partly caused by ischaemia/reperfusion injury (I/R) (1). The damage induced by I/R results in instability and impairment of detrusor contractility during micturition. In recent years, several *in vitro* studies have indicated that female organs (such as brain and heart) are less vulnerable to I/R than those of male, and suggested 17 $\beta$ -estradiol as the critical factor that allows protection. On this basis, we investigated the effects of anoxia-glucopenia (A-G) on both male and female rodent's urinary bladder and the role of estrogens as neuroprotective agents.

Detrusor strips and whole rodent's bladders were exposed to 1 and 2 h of A-G and subsequent period of reperfusion. Intrinsic nerves underwent electrical field stimulation (EFS); smooth muscle was stimulated with carbachol (10  $\mu$ M) and high potassium (60 mM). The effect of 17 $\beta$ -estradiol on the contractile response and the ensuing recovery in normal Krebs' solution was monitored adding it to the bath medium, at different concentrations (0.1, 1, 3, 10, and 30  $\mu$ M), 60 min before applying A-G (pre-incubation), during the A-G, and in the first 30 min of reperfusion period. Furthermore, on both male and female rats, *in vivo* treatments with 17 $\beta$ -estradiol (50  $\mu$ g/kg) and anastrozole (20 mg/kg), an aromatase II inhibitor, were performed. To image Ca<sup>2+</sup> dynamics, detrusor strips from male rats were exposed to 10  $\mu$ M oregon green 488 BAPTA-1 AM for 90 min, and then image series were acquired with laser-scanning confocal microscope.

17 $\beta$ -Estradiol by itself decreased EFS responses in a concentration-dependent manner. The recovery of female urinary bladder nerve responses after 1 h and 2 h of A-G were significantly higher than those of male. Moreover, 1  $\mu$ M 17 $\beta$ -estradiol exerted a neuroprotective effect in male rat urinary bladder subjected to A-G/reperfusion. The neuroprotective role of estrogens was confirmed either by *in vivo* treatments with 17 $\beta$ -estradiol, which significantly improved EFS responses as compared to control, or with anastrozole, which decreased EFS responses of about 30%. Global Ca<sup>2+</sup> flashes, which represent Ca<sup>2+</sup> influx through voltage-dependent Ca<sup>2+</sup> channels during action potential (2) were recorded. In the tissues subjected to A-G/reperfusion, the amplitude of global flashes was much higher than in the non-ischaemic controls, while the frequency was slightly higher but not statistically significant. Following A-G/reperfusion, the frequency of Ca<sup>2+</sup> flashes in strips treated with high concentrations of 17 $\beta$ -estradiol (10 and 30  $\mu$ M) was significantly increased, while the amplitude was significantly decreased.

A higher susceptibility to ischaemic damage of male rodent urinary bladder nerves compared to female ones has been demonstrated. 17 $\beta$ -Estradiol by itself showed relaxant effects through non genomic pathway in male and female detrusor. Moreover, in urinary bladders subjected to A-G/reperfusion damage, 1  $\mu$ M 17 $\beta$ -estradiol exerted neuroprotective properties.

## References:

1. Brading AF, Pessina F, Esposito L, Symes S (2004) Scand J Urol Nephrol Suppl (215): 84-92.
2. Heppner TJ, Bonev AD, Nelson MT (2005) J Physiol 564: 201-12.



**PHYTOESTROGEN GENISTEIN REDUCES NOCICEPTIVE  
HYPERSENSITIVITY IN THE MOUSE NEUROPATHY MODEL:  
INVOLVED MECHANISMS**

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The neuroprotective effects of estrogens were demonstrated in a variety of neurodegenerative and neuroinflammatory diseases. There is great interest in soy isoflavones as a potential alternative to the synthetic selective estrogen receptor modulators for therapeutic use, not restricted only to hormonal ailments, but also in inflammatory and ischemic disorders. The first aim of the present work was to investigate the effect on neuropathic pain of genistein (4',5-7 trihydroxyisoflavone), a nutraceutical present in soybean. This compound shares structural features with the potent endogen 17 $\beta$ -estradiol. These features confer ability to bind estrogen receptors (ER); in fact, genistein binds both ER $\alpha$  and ER $\beta$ , with higher affinity for ER $\beta$ , expressed also in neuronal and immune cells.

Painful neuropathy was induced in C57BL/6J male mice by sciatic nerve chronic constriction injury. Responses to nociceptive stimuli were measured before, 3, 7, and 14 days after the surgical procedure. Heat hypersensitivity (thermal hyperalgesia) was assayed using Plantar test and mechanical allodynia by Dynamic Plantar Aesthesiometer. The phytoestrogen genistein (1, 3, and 7.5 mg/kg, s.c.), administered to neuropathic mice, once a day, for 11 days, starting from 3<sup>rd</sup> day after injury, reversed the nociceptive hypersensitivity in a time- and dose-dependent fashion. The activation of classical nuclear ER, the interaction with membrane binding sites, the antioxidant effects and the modulation of neurotransmission mediate the effects of the estrogens. So, the second objective of this work was to investigate the mechanisms underlying the antiallodynic and antihyperalgesic ability of genistein. To study whether the effect of genistein was ER-mediated, neuropathic mice were treated with an ER antagonist, ICI 182,780, co-administrated (25 mg/kg, s.c.) with genistein (3 mg/kg), twice a day, for 3 days, from the 11<sup>th</sup> day after the nerve injury. ICI 182,780 deleted the antiallodynic but not the antihyperalgesic effect of genistein. The oxidative stress, the NO/NOS system and the NF- $\kappa$ B pathway are involved in the neuropathic pain development and maintenance. To characterize the other mechanisms involved in pain relief induced by genistein, we measured the levels of free radicals (ROS) and the products of lipid peroxidation (malondialdehyde, MDA) through fluorimetric and spectrophotometric procedure, respectively. The increase in both ROS and MDA in the hind paws, ipsilateral to nerve injury, was reduced by repeated effective treatment with the isoflavon. In sciatic nerve, L4-L6 dorsal root ganglia, ipsilateral to surgery, L4-L6 dorsal spinal cord, and finally, ipsi- and contra-lateral thalamus, the content of inducible and neuronal isoforms of NOS was determined through Western blot analysis. The phytoestrogen decreased the overproduction of two NOS isoforms, at level of both peripheral and central nervous system. We also evaluated if the genistein efficacy could be attributed to the modulation of the NF- $\kappa$ B activation both peripherally (sciatic nerve) and centrally (L4-L6 dorsal spinal cord), using an ELISA assay. In ligated sciatic nerve, the NF- $\kappa$ B activation was reversed by phytoestrogen treatment. The genistein efficacy on pain hypersensitivity could be attributed partially to ER-dependent activity, but also to the decrease in oxidative stress and the reduction in the NO/NOS system and peripheral NF- $\kappa$ B activation.

## INFLUENCE OF MEDIA, MEDICAL JOURNALS AND REGULATORY MEASURES ON THE RATE OF SPONTANEOUS ADR REPORTING

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The reporting of adverse drug reactions (ADRs) by doctors and other health personnel is the mainstay of the post-marketing surveillance systems. Underreporting and selective reporting are considered the main limitations of a spontaneous reporting-based pharmacovigilance system. However, also an excessive reporting, induced by external influences, may impair the signal detection, by increasing the noise level.

The aim of this study was to assess the influence of external factors (media, medical journals and regulatory decisions ) on the rate of adverse drug reaction reporting. We focused on four typical situations occurred in Italy and/or Europe as illustrative of four different scenarios: ACE inhibitors-induced cough and restriction of angiotensin receptor blockers (ARB) reimbursement, statins and rhabdomyolysis, nimesulide and hepatic toxicity, and coxibs and cardiovascular risk.

The study was based on data coming from spontaneous reporting in six Italian regions collected from January 1995 to December 2005. We analysed a 10 year period, as a reasonable time interval around the considered events highlighting the influence of external factors on the rate of ADR reporting. Drug sales data were also considered to assess the possible changes of drug use. Sales data were expressed as daily defined dose (DDD) per 1000 inhabitants per day.

ACE inhibitors: an increase of about four-fold in the number of reports of ACE inhibitor-induced cough was observed in 1998-1999 following the restriction of ARB reimbursement. In the same period sales of ARB strongly increased whereas ACE inhibitors increased only slightly. Statins: the percent of ADRs of rhabdomyolysis increased about 5-fold after the “Lipobay scandal” in 2001 and progressively decreased in the following years. The sales of these drugs increased from 2000 to 2005. Nimesulide: an increase of hepatic ADR reporting was observed after withdrawal of the drug from Finnish and Spanish markets in 2002, without any effects on sales. Coxibs: a modest increase of cardiovascular ADR reporting was observed after rofecoxib withdrawal in 2004, with a sharp drop of both sales and ADR reports in 2005.

Post-marketing pharmacovigilance systems play a key role in the assessment of drug risk/benefit profile, however underreporting, selective reporting and the external influences can jeopardize the early detection of new signals and bias their interpretation. This analysis also emphasised the importance of adopting appropriate risk communication strategies as a crucial part of health policy, agreed by health regulatory body, drug manufacturers and doctors, ensuring in the meantime independent and appropriate information to the patients.

## **M<sub>3</sub> MUSCARINIC RECEPTORS IN PERIPHERAL LUNG PARENCHYMA OF PATIENTS WITH CHRONIC OBSTRUCTIVE PULMONARY DISEASE**

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Chronic obstructive pulmonary disease (COPD) is a disease state characterized by airflow limitation that is not fully reversible and is usually progressive, together with abnormal inflammatory responses. COPD is the fourth leading cause of mortality worldwide and the main cause is cigarette smoking. The aim of this study was to investigate the presence of M<sub>3</sub> muscarinic receptors in peripheral lung parenchyma in COPD patients and to compare these data with those obtained analysing age-matched smokers with normal lung function (control subjects). In order to evaluate whether the difference between COPD patients and control subjects was specific for the M<sub>3</sub> receptor subtype, we have studied the presence of another receptor, the  $\beta$ 2 adrenergic receptor in the same subjects.

Saturation binding experiments for M<sub>3</sub> receptor were performed with [<sup>3</sup>H]-QNB (0.02-2 nM) in the presence of methoctramine (50 nM) and atropine 1  $\mu$ M for unspecific binding. Saturation binding experiments for  $\beta$ 2 receptor were performed with [<sup>3</sup>H]-CGP12177 (0.02-2 nM) in the presence of 1  $\mu$ M CGP20712A and 10  $\mu$ M propranolol for unspecific binding.

The affinity of M<sub>3</sub> receptors was similar in COPD patients compared with control group ( $K_D = 0.20 \pm 0.01$  nM and  $0.19 \pm 0.02$  nM, respectively). On the contrary the receptor density ( $B_{max}$ ) expressed as fmol/mg protein was significantly increased in COPD patients compared with the control subjects ( $33 \pm 3$  fmol/mg protein and  $16 \pm 2$  fmol/mg protein, respectively). The affinity and density of  $\beta$ 2 receptor were similar in COPD patients compared with control group.

These data show an increase of M<sub>3</sub> muscarinic receptors expressed in lung parenchyma of COPD patients suggesting important implications for anticholinergic therapy in the future by using selective drugs with a lower profile of side effects.

**PERIAQUEDUCTAL GREY METABOTROPIC GLUTAMATE RECEPTOR SUBTYPE 7 AND 8 MEDIATE OPPOSITE EFFECTS ON AMINO ACID RELEASE, ROSTRAL VENTROMEDIAL MEDULLA CELL ACTIVITIES, AND THERMAL NOCICEPTION.**

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The current study has investigated the involvement of periaqueductal grey (PAG) metabotropic glutamate subtype 7 (mGlu<sub>7</sub>) and 8 (mGlu<sub>8</sub>) receptors in modulating rostral ventromedial medulla (RVM) ongoing and tail flick-related ON and OFF cell activities. Our study has also investigated the role of PAG mGlu<sub>7</sub> receptor on thermoceptive threshold and PAG glutamate and GABA release. Intra-ventrolateral PAG (S)-3,4-DCPG (2 and 4 nmol/rat) or AMN082 (1 and 2 nmol/rat), selective mGlu<sub>8</sub> and mGlu<sub>7</sub> receptor agonists, respectively, caused opposite effects on the ongoing RVM ON and OFF cell activities. Tail flick latency was increased or decreased by (S)-3,4-DCPG or AMN082 (2 nmol/rat), respectively. (S)-3,4-DCPG reduced the pause and delayed the onset of the OFF cell pause. Conversely, AMN082 increased the pause and shortened the onset of OFF cell pause. (S)-3,4-DCPG or AMN082 did not change the tail flick-induced onset of ON-cell peak firing. The tail flick latency and its related electrophysiological effects induced by (S)-3,4-DCPG or AMN082 were prevented by MSOP (100 nmol/rat), a group III mGlu receptor antagonist. Intra-ventrolateral PAG perfusion with AMN082 (10 and 25 μM), decreased thermoceptive thresholds and glutamate extracellular levels. A decrease in GABA release was also observed. These results show that stimulation of PAG mGlu<sub>8</sub> or mGlu<sub>7</sub> receptors could either relieve or worsen pain perception. The opposite effects on pain behaviour correlate with the opposite roles played by mGlu<sub>7</sub> and mGlu<sub>8</sub> receptors on glutamate and GABA release and the ongoing and tail flick-related activities of the RVM ON and OFF cells.

## NEUROPROTECTIVE EFFECT OF GUANOSINE IN HUMAN NEUROBLASTOMA SH-SY5Y CELLS

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Guanosine is a purine nucleoside that, like its adenine-based counterpart adenosine, shows a spectrum of biological activities. We have previously demonstrated that guanosine exerts neuroprotective effect on a neuroblastoma cell line (SH-SY5Y) under stress condition. In fact, guanosine increased in a concentration- and time-dependent manner cell viability after serum deprivation (0% FBS). These data, obtained by MTT test, have been confirmed by cell flow cytometry. Indeed, guanosine decreased the percentage on G<sub>0</sub> phase whereas increased the percentage on S phase of serum-deprived cells. One of the mechanisms possibly involved in the guanosine-induced neuroprotective effect is the activation of MAPK cascade, since guanosine induced a time- and dose-dependent phosphorylation of ERK1/2, measured by CASE TM Kit (cellular activation of signalling ELISA).

Once established the neuroprotective effect of guanosine in stressed neurons, we focussed our attention on guanosine-induced morphological changes in cells cultured in the presence of 10% FBS.

Guanosine did not affect viability in unstressed SH-SY5Y cells but it was able to induce differentiation as based on the evaluation of neurite extension that is one of the major and essential markers for neuronal differentiation. SH-SY5Y cells treated with different concentration of guanosine (10-150  $\mu$ M) for increasing time (1-3-7 days), were fixed with methanol and cell images were acquired with Leica IM computerized program. Retinoic acid was used as positive control. Cells were considered differentiated if they had at least one process longer than the cell body. The ability of guanosine to enhance neurite extension was time- and dose-dependent. Furthermore, the neurite lengths after the different treatments were measured by the computerized program Scion Image. Additional experiments will be performed to better characterize these effect quantifying specific markers expression by immunocytochemistry.

Since differentiation and cell growth are mutually exclusive phenomena, we analysed cell proliferation by colouring a cell membrane protein with a specific dye (sulforhodamine B assay).

Our results confirmed that there is a time- and dose-dependent decrease in cell proliferation after guanosine treatments (IC<sub>50</sub> 147 $\pm$ 47  $\mu$ M), as well as after exposure to retinoic acid.

In the attempt to identify the mechanisms that underlie guanosine-induced differentiation, we investigated different signal transduction pathways. First, we demonstrated that guanosine (30-300  $\mu$ M) dose- and time-dependently activates ERK1/2 MAP kinases with a peak at 2.5 min (100  $\mu$ M). Then we quantified intracellular cAMP production by competition experiments versus [<sup>3</sup>H]cAMP and we analyzed [Ca<sup>2+</sup>]<sub>i</sub> movement for 15 min after guanosine addition (100  $\mu$ M-1 mM) by imaging method, loading neuroblastoma cells with FURA-2. These important signal transduction pathways are slightly stimulated by guanosine. We will investigate the involvement of the PI3K/Akt cascade.

An important point is to ascertain if guanosine effects are mediated by the activation of guanosine-specific receptors. To achieve this goal, we preliminary characterized guanosine transport on neuroblastoma cells. These cells constitutively express equilibrative nucleoside transporters, as demonstrated by the presence of saturable and reversible binding sites for [<sup>3</sup>H]NBTI on intact SH-SY5Y cells (K<sub>d</sub> 1.5 $\pm$ 0.2 nM). [<sup>3</sup>H]Guanosine and [<sup>3</sup>H]adenosine uptake through this NBTI-sensitive equilibrative transporter occurs with similar kinetic but different affinity, guanosine uptake being 100 fold less than adenosine.

Our results, with the knowledge of the high levels of extracellular guanosine in central nervous system, suggest for guanosine a main role on neuronal plasticity.

## EFFECT OF DIAZEPAM ON COCATROPINE-INDUCED TOXICITY

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At the end of 2004 and the beginning of 2005, a number of cases of intoxications were reported in Belgium, France, Italy, and the Netherlands, involving a cocaine/atropine mixture known as cocatropine (1). Operators of emergency units reported the following symptoms: excitement or restlessness, psychotic symptoms, hallucinations, seizures, reduced consciousness, tachycardia, hypertension, respiratory failure, dry mouth and severe mydriasis. One fatal case has been notified in Italy. Very little information is available about the effect of concomitant cocaine and atropine in animals and the possible antidotic treatment. In only one report (2) atropine enhanced the central respiratory toxicity of cocaine by acting synergistically at caudal chemosensitive areas of the ventrolateral *medulla oblongata* in urethane-anesthetized and tracheotomised cats.

On this basis, the present study was designed to investigate a possible antidote against cocatropine-induced toxicity in Wistar rats. Thus, we studied the effects of cocaine (40 mg/kg) and atropine (20 and 60 mg/kg) on physiological, electroencephalographic (EEG), and behavioural parameters after acute or repeated treatment.

Seizures (latency to the first and score), death, locomotor activity evaluated in an activity cage, rectal temperature, and EEG mean total spectral power evaluated by PowerLab System, were assessed for 60 min. Heart rate and blood pressure were continuously monitored, through a pressure transducer, in anesthetized rats for 120 min. Diazepam, chosen as possible antidotic drug, was given after the onset of the first seizure.

Atropine (20 and 60 mg/kg) or cocaine (40 mg/kg) alone did not induce any seizure or death, but the combination significantly increased both, after acute and binge treatment. There was a significant increase of EEG mean total spectral power in cocatropine group in comparison with cocaine-treated animals. Hyperlocomotion was observed in non seizing rats treated with cocaine or cocatropine. Cocaine, atropine 60, and cocatropine (40+20 and 40+60) treated animals showed hyperthermic effects if non-seizing, while, if seizing, hypothermic effects. An initial hypertensive and tachycardic effect, within 15 min, was followed by a secondary fall in the cocatropine (40+60) group. Diazepam (5 mg/kg) partially or fully reversed cocatropine-induced toxicity.

In conclusion, an increased toxicity induced by cocaine adulterated with atropine in comparison to cocaine alone, given acutely or repeatedly to rats, in proportions similar to those encountered in cases of human intoxication, was demonstrated. These findings provide detailed evidence of a synergistic toxic effect of the two drugs combined on behavioural, EEG, and physiological parameters. Diazepam appears to be effective against this toxicity, suggesting a useful potential strategy for dealing with cocatropine-related hospital emergencies.

### References:

1. Sedefov R (2005) Drugnet Europe (European Monitoring Center for Drugs and Drug Addiction) April/June: 6
2. Dehkordi O, et al. (2001) Life Sci 69: 2017-25.

**HERPES SIMPLEX VIRUS-1 INFECTION ALTERS RAT SMALL INTESTINAL MOTILITY****Zoppellaro Chiara**

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Infection by pathogens is considered to play a role in irritable bowel syndrome (IBS), a widespread gastrointestinal (GI) disorder (1). Among common pathogens, herpes simplex virus-1 (HSV-1), orally inoculated to laboratory animals, targets neurons in the enteric nervous system (ENS) and it is involved in the pathogenesis of GI disorders (2). The aims of the present study were to establish an *in vivo* model of HSV-1 infection in the rat intestine and to evaluate possible effects of this virus on intestinal motility. Chronic HSV-1 infection was established by inoculating rats intranasally ( $10^3$  pfu) and again intragastrically (i.g.,  $10^8$  pfu) 4 weeks (wk) later. Infected or mock infected rats were sacrificed 1 and 6 wk after the i.g. inoculation. The presence of HSV-1 infection was determined by PCR amplification of HSV-1-tk gene, RT-PCR for HSV-1 latency associated transcripts (LATs) and early gene ICP-4, in situ hybridization, and immunofluorescence for glycoprotein-C (gC). In isolated ileum and duodenum segments (2 cm), mounted vertically in organ baths, changes in muscle tension were recorded using isometric transducers. Concentration-response curves to carbachol (0.01-100  $\mu$ M) and to KCl (3-80 mM) were obtained cumulatively. On ileum preparations isolated from infected and control rats, electrical field stimulation (EFS) experiments were also performed using platinum electrodes connected to a Grass S88 stimulator. Frequency-response curves (2-40 Hz, 0.1 ms pulse duration, 10 s pulse trains) were constructed in the presence or absence of tetrodotoxin (TTX, 1  $\mu$ M) and atropine (1  $\mu$ M). Both controls and HSV-1 infected rats did not show clinical and histological abnormalities in the GI tract. In the brain, full-thickness gut, and isolated neurons a latent HSV1 infection was shown by the presence of viral tk-DNA and LAT mRNA and by the absence of ICP-4 mRNA transcripts and HSV-1gC immunoreactivity, whereas in situ hybridization showed HSV-1 DNA in central nervous system and ENS.  $E_{max}$  values for carbachol-induced contractions were significantly increased by 34% and 56% at 1 wk and 6 wk postinfection (PI) in the ileum and by 121% in the duodenum at 1 wk PI. Maximum response to KCl was reduced by 46% in the ileum and by 35% in the duodenum at 1 wk. Moreover in ileum preparations the responses to EFS were significantly increased after 1 wk PI compared to controls. Following i.g. delivery, HSV-1 establishes a latent infection in the ENS and also significantly affects intestinal smooth muscle responsiveness to membrane depolarization by KCl while increasing neurally mediated responses and its sensitivity to muscarinic receptor stimulation in this time frame.

## References:

1. Lea R, Whorwell PJ (2002) Gut 51: 305-6.
2. Gesser RM, Koo SC (1996) J Virol 70: 4097-102.

## METABOLIC DYSFUNCTIONS IN ISOLATION-REARED RATS

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Metabolic syndrome is a cluster of disorders comprising obesity, dyslipidaemias, glucose intolerance, insulin resistance and hypertension. There are evidences that associations between schizophrenia and these disorders actually exist. Schizophrenic drug-naïve, first episode patients have increased visceral fat deposition and show evidence of basal overactivity of the pituitary-adrenal axis when compared to control subjects (1). Epidemiological data suggest that stress is linked to the development of overweight, obesity and metabolic disease (2) but there are limited animal models to allow investigation of the aetiology of increased adiposity resulting from exposure to stress.

The aim of this study was to investigate if an animal model of schizophrenia can mirror the consequences of stress in humans. In particular, we examined the effects of social isolation rearing, a non-pharmacological method of inducing schizophrenia-like behavioural deficits (3), on body weight, visceral fat, and food intake. Either plasma levels of glucose, insulin, adiponectin, and lipidic profile or salivary alpha-amylase and cortisol were also recorded from Wistar isolated rats (ISO) and compared with control groups (GRP).

Our results showed that social stress resulted in significant effects on body composition, but not on totally body weight, such that ISO progressively developed characteristics of obesity that occurred, in part, through neuroendocrine alterations, changes in food intake amount, and accumulation of fat in visceral adipose tissues. Our data also suggested an impaired fasting glucose tolerance in male GRP animals.

Basal levels of glucose, insulin, adiponectin and triglycerides did not differ between the two groups of animals. We have found that ISO showed lower amylase and higher cortisol levels compared to GRP. In conclusion our results provide evidence that isolation-reared rats produced metabolic dysfunctions in Wistar rats.

### References:

1. Ryan M, Thakore J (2002) *Life Sciences* 71: 239-57.
2. Tamashiro KL, et al. (2007) *Physiol Behav* doi:10.1016 [Epub ahead of print].
3. Leng A, Feldon J (2004) *Pharmacol Biochem Behav* 77: 371-9.