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ROLE OF GLUCOSE-6 PHOSPHATE DEHYDROGENASE (G6PD) IN REGULATING INFLUENZA VIRUS REPLICATION AND HOST RESPONSE TO INFECTION

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The aim of this project is to deepen the molecular mechanisms underlying the regulation of G6PD expression and activity during influenza virus infection and to evaluate whether modulation of this enzyme contributes to virus-induced oxidative stress and to increased viral yield.
Influenza virus replicates into the cell exploiting several pathways involved in the regulation of host responses. The outcome and the severity of the infection are thus strongly conditioned by multiple host factors, including age, sex, metabolic and redox conditions of the target cells. During infection, oxidative stress, mainly determined by an increase of ROS production and decrease of intracellular glutathione (GSH) content, activates many cell pathways used by the virus to complete its life-cycle. Glucose-6-phosphate dehydrogenase (G6PD), the first and the rate-limiting enzyme of pentose phosphate pathway, is also responsible for the production of reducing equivalents in the form of NADPH, which is used for regenerating the reduced form of GSH and re-establishing physiologic redox conditions. G6PD-deficiency has been correlated with increased infectivity and severity of different viral infections and our preliminary results on influenza virus have shown that G6PD expression and activity were down-regulated by the virus.
In the first period of the present proposal, our activities were focused to study viral replication in G6PD silenced cells and to evaluate whether G6PD enzyme modulation contributes to the redox unbalance of infected cells.
Our results demonstrated that human lung carcinoma cells (A549), silenced for G6PD (Isi cells) by the small-interference RNA assay and subsequently infected with the influenza virus A PR8/H1N1, significantly produced more viral particles than not silenced cells (I cells). Indeed, all viral proteins analyzed by western blot were highly expressed in Isi cells and viral titer, measured by TCID50 (Tissue Culture Infectious dose 50%) in the supernatants of infected cells, was more than 1 log higher in Isi cells compared to that found in I cells. These results indicated a key role of G6PD in the regulation of influenza virus replication. To verify whether virus-induced down-

regulation of G6PD enzyme contributes to the redox changes in infected cells, we measured GSH levels in silenced and not silenced cells. As expected, we found a significant reduction of GSH levels in infected cells compared with uninfected (Ctr) cells; the GSH decrease was more pronounced in Isi cells with respect to silenced Ctr cells, thus confirming G6PD contribution in regulating intracellular redox state during infection. Furthermore, infected cells were analyzed for the expression of the transcription factor Nrf2, which in conditions of oxidative stress transcribes for genes of the antioxidant response including G6PD and enzymes responsible for the synthesis of GSH. We found a strong inhibition of Nrf2 expression that was correlated with the reduction of G6PD expression. Furthermore, also in G6PD silenced cells there was an inhibition of Nrf2 expression, indicating a fine cross-talk between Nrf2 pathway and G6PD enzyme.
All the data demonstrate another mechanism through which the virus interferes with the antioxidant response of the cell to maintain oxidative conditions into the cell that are fruitful for its replication.
In the next year of the project, we aim to deepen the mechanism underlying the virus-induced inhibition of G6PD. Results obtained will provide new knowledge on the correlation between G6PD-deficiency, redox state alterations and viral replication. A targeted therapy aimed at restoring reducing conditions in G6PD-deficient patients may contribute to control the progression and the outcome of viral infection.

Publications

Simona Anticoli, Donatella Amatore, Paola Matarrese, Marta De Angelis, Anna Teresa Palamara, Lucia Nencioni*, Anna Ruggieri*. Counteraction of HCV-Induced Oxidative Stress Concurs to Establish Chronic Infection in Liver Cell Cultures. Oxidative Medicine and Cellular Longevity 2019, ID 6452390 IF: 4.936

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Bruno Mattia Bizzarri*, Angelica Fanelli, Davide Piccinino, Marta De Angelis, Camilla Dolfa, Anna Teresa Palamara, Lucia Nencioni*, Claudio Zippilli, Marcello Crucianelli, Raffaele Saladino. Synthesis of Stilbene and Chalcone Inhibitors of Influenza A Virus by SBA-15 Supported Hoveyda-Grubbs Metathesis. Catalysts 2019, 9 IF: 3.465

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THE ROLE OF METABOTROPIC GLUTAMATE RECEPTOR 1 IN METHAMPHETAMINE CRAVING RELAPSE: FROM MECHANISMS TO STRATEGIES FOR THERAPEUTIC INTERVENTION

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Relapse to methamphetamine (Meth) use can occur even after prolonged abstinence and is often precipitated by exposure to drug-associated cues.

On the basis of clinical observations, Gawin and Kleber proposed that cue-induced cocaine craving increases during early abstinence and remains elevated for extended time periods. An analogous phenomenon, termed 'incubation of drug craving' has been observed in rats trained to self-administer (SA) several drugs of abuse, including Meth. Clinical studies have confirmed that incubation of craving also occurs in Meth drug users. The demonstration of incubation in human studies provides support for the translational potential of therapeutic targets for relapse uncovered through rodent mechanistic studies.

From an animal model-to-human translational perspective, a limitation of rodent incubation of craving studies is that the abstinence period preceding the relapse tests is forced (experimenter-imposed), and achieved by removing the subjects from the drug self-administration environment. In contrast, in humans, abstinence is often voluntary due to either the negative consequences of chronic drug use or the availability of competing alternative incentives. In the latter case, relapse vulnerability is high after the loss of the alternative incentives. Based on these considerations, we have recently developed a choice-based rat model of relapse after voluntary abstinence. Notably, the proposed model of this application mimics human relapse after cessation of successful contingency management (CM) where the availability of monetary vouchers, given in exchange for 'clean' urine samples, maintains abstinence. In this choice-based rat model, rats with a history of palatable food and extended access Meth self-administration would voluntary abstain from drug self-administration when given a mutually exclusive choice between the two rewards. In this study, the model was used to demonstrate that time-dependent increases in Meth seeking (incubation of Meth craving); occur after voluntary abstinence when the alternative non-drug reward was discontinued. This aspect has relevance because a recent report on the effectiveness of varying the duration of the CM

intervention on Meth addicts, revealed that the number of participants who remained abstinent increased as the exposure to CM increased.

Speculatively, these findings suggest that the reduced propensity to relapse may be mediated by a CM-induced attenuation of 'incubated' Meth craving, which represent the backbone of this proposal. Indeed, in this proposal we contrast the forced and voluntary abstinence animal models of drug craving, and investigate similarities and differences in the synaptic mechanisms underlying Meth craving.

1) Behavioural results: Here we confirm (relative to the preliminary results provided) that the magnitude over time of "incubated" Meth craving is different between the forced and voluntary abstinence condition. In particular, we observed: 1) A similar rising phase between the forced and voluntary abstinence condition. Specifically, the number of non-reinforced lever pressing on day 7 and day 21 were significantly higher from day1 for both the forced and voluntary abstinence groups; 2) A steeper falling phase in the voluntary abstinence relative to the forced abstinence condition (abstinence day 35 was significantly higher in the forced abstinence group relative to the voluntary group. These data may account for the human findings described above and suggest the protective effect on craving and relapse of extended, as opposed to short, CM based therapies.

In this proposal, (at a neurobiological level) we are targeting the NAc because changes in neuronal activity in the core, but not in the shell, correlate with the incubation of Meth craving in the forced abstinence and voluntary abstinence procedures.
In drug-naïve adult rats, GluA2-containing Ca2+-impermeable AMPARs (CI-AMPARs) mediate most excitatory transmission onto MSNs. However, after ~1 month of abstinence from extended-access cocaine self-administration, higher conductance GluA2-lacking Ca2+-permeable AMPARs (CP-AMPARs) accumulate in NAc core synapses to drive MSNs and the expression of 'incubated' cocaine craving. The objective is to determine if this also holds for Meth under both abstinence conditions. Determining the subunit composition of CP-AMPARs is essential for understanding mechanisms that underlie their accumulation. Indeed, homomeric GluA1 and GluA3 receptors will be differently regulated by phosphorylation or scaffolding proteins due to marked differences in the C termini of GluA1 vs GluA3. Our working hypothesis was: 1) 'Meth incubated rats' in both forced and voluntary abstinence condition exhibit increased homomeric GluA1 receptors in NAc core during the rising phase (AD7) of the incubation of Meth craving; 2) the attenuation of incubation of Meth craving observed by AD35 in the voluntary abstinence group, but not in the forced group, is associated to the elimination of homomeric GluA1 CP-AMPARs.

2) Biochemical results: Quite surprisingly, we observed no significant changes between groups in surface or total GluA1, GluA2 and GluA3 subunits relative to the saline control, and abstinence days (1, 7 and 35).

Future directions: Based on the results reported above (in conflict with our predictions), we planned (for the next 6 months) to assess whether GluA1 translation is affected during the voluntary abstinence condition. We know that this stands for the forced abstinence condition (Murray et al., 2019). Indeed, our methods could not have had the needed resolution to detect more subtle biochemical changes putatively occurring at a synapse level.

Publications

(in preparation) Factors that modulate incubation of drug craving and their clinical implications. – Invited review – Neuroscience and Biobehavioural Review (special issue for FENS 2020; in preparation) IF 8.002

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MITOCHONDRIAL tRNA RELATED DISEASES: IMPLEMENTATION OF CELLULAR MODELS TO EVALUATE THE TISSUE-SPECIFIC EFFECTS AND RESCUING MECHANISMS OF THERAPEUTIC MOLECULES

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Mutations in genes coding for mitochondrial (mt)-tRNAs (MTTs) are responsible for a wide range of currently untreatable pathologies. Previous studies on the mechanisms underlining mt-tRNA associated diseases have been performed on cells which can be easily obtained from patients, such as fibroblasts, and the *trans*-mitochondrial hybrid model (cybrid). As compared to fibroblasts, cybrid cells have the advantage to be available in large amounts at low costs. However, being undifferentiated neoplastic cells, they do not entirely reproduce the biochemical phenotype observed in patients' most seriously affected tissues, for instance heart, muscle and brain. The possibility to develop cellular models able to recapitulate the tissue-related effects of mt-DNA mutations has been recently opened up by the production of induced pluripotent stem cells (iPSCs) obtained from patients bearing mt-tRNA point-mutations (1, 2).
Accordingly, the aims of our project are:

- to develop neurons, cardiomyocytes and myotubes from iPSCs obtained from patients carrying either the homoplasmic mutation m.4277T>C in mt-tRNA^{Ile} gene (causing mitochondrial cardiomyopathy) or the heteroplasmic mutations m.3243A>G in mt-tRNA$^{Leu(UUR)}$ gene and m.8344A>G in mt-tRNA^{Lys} gene (causing severe mitochondrial syndromes);
- to analyze the pathologic phenotype of iPSCs and iPSC-derived cells;
- to evaluate the possible rescuing effect of therapeutic molecules on these cellular models.

We have already available in our laboratory iPSCs bearing all the mt-tRNA point mutations listed above.
In the first months of this project, we progressed with: i) the phenotype evaluation of iPSCs carrying each single mitochondrial mutation and ii) the generation of iPSCs derived cells (neural stem cells and cardiomyocytes).
<u>Evaluation of iPSCs phenotype</u>
The evaluation of the mutated iPSCs phenotype was based on cell viability and apoptotic rate, as well as on parameters more strictly related to mitochondrial function: ATP production, oxygen consumption, ROS production, levels of intracellular lactate.
We first assessed the viability of mutant iPSCs. Briefly, we seeded 10^5 iPSCs in

Essential 8 medium for 24h, and then, by using the CyQUANT cell proliferation assay kit (Molecular Probes, Invitrogen) we compared the viability of mutant and wild type cells. Our preliminary results showed a decreased viability of all mutant as compared to wild type iPSCs (Figure 1A).
We then evaluated the cellular ATP production of mutant iPSCs cultured in Essential 8 medium by using the ATPlite Assay system (PerkinElmer) according to the manufacturer instructions. Luminescence intensity was quantified and normalized by the number of cells for each well. Our preliminary results suggest a decreased ATP production in iPSCs bearing both m.3243A>G and m.4277T>C (Figure 1B).

Generation of iPSCs derived cells

After iPSCs characterization, we generated neural stem cells (NSC) bearing the m.3243A>G, m.8344A>G or m.4277T>C mutation, according to established protocols (1, 3) and confirmed their phenotype by immunofluorescence (Figure 2A). By sequence analysis of whole mtDNA we excluded presence of further mtDNA mutations or depletion/major rearrangements, possibly induced by the differentiation process (data not shown). Furthermore, we showed by RFLP analysis (4, 5, 6) that the mutation load did not change significantly upon differentiation (data not shown).
In parallel we generated cardiomyocytes (CM) bearing the m.3243A>G mutation. For cardiac differentiation we used the PSC Cardiomyocyte Differentiation Kit (Life Technologies, Thermo Fisher scientific) following the manufacturer's instructions. Briefly, we seeded $3x10^4$ iPSCs in Essential 8 Medium on Geltrex matrix-coated plate. At 70% of confluency (2–3 days) the medium is changed to Cardiomyocyte Differentiation Medium A to start cardiac induction. Medium A is replaced with Medium B after two days, and with Medium C following 2 more days for final differentiation. At day 9 we observed contracting cardiomyocytes (MS-CM). To confirm their phenotype, we performed an immunofluorescence analysis by using Human Cardiomyocyte Immunocytochemistry Kit (Invitrogen, Thermo Fisher scientific) (Figure 2B). We are currently performing both mtDNA analysis and evaluation of heteroplasmy of MS-CM.

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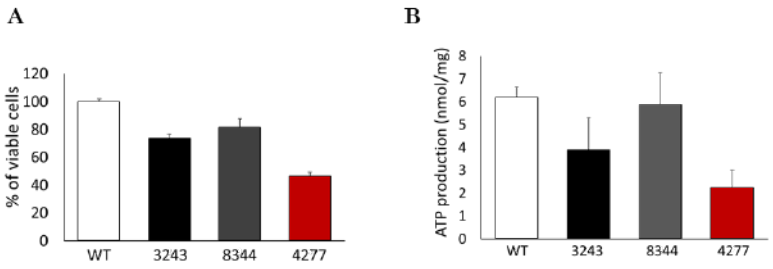

Figure 1 Analysis of phenotype of iPSCs bearing different mt-tRNA point mutations
A. Mutant iPSCs showed a decrease in cell viability in comparison with control iPSC (wild type, WT), when cultured in Essential 8 medium.
B. Both 3243 and 4277 iPSCs showed a decreased ATP production in comparison with control iPSC (wild type, WT), when cultured in Essential 8 medium.

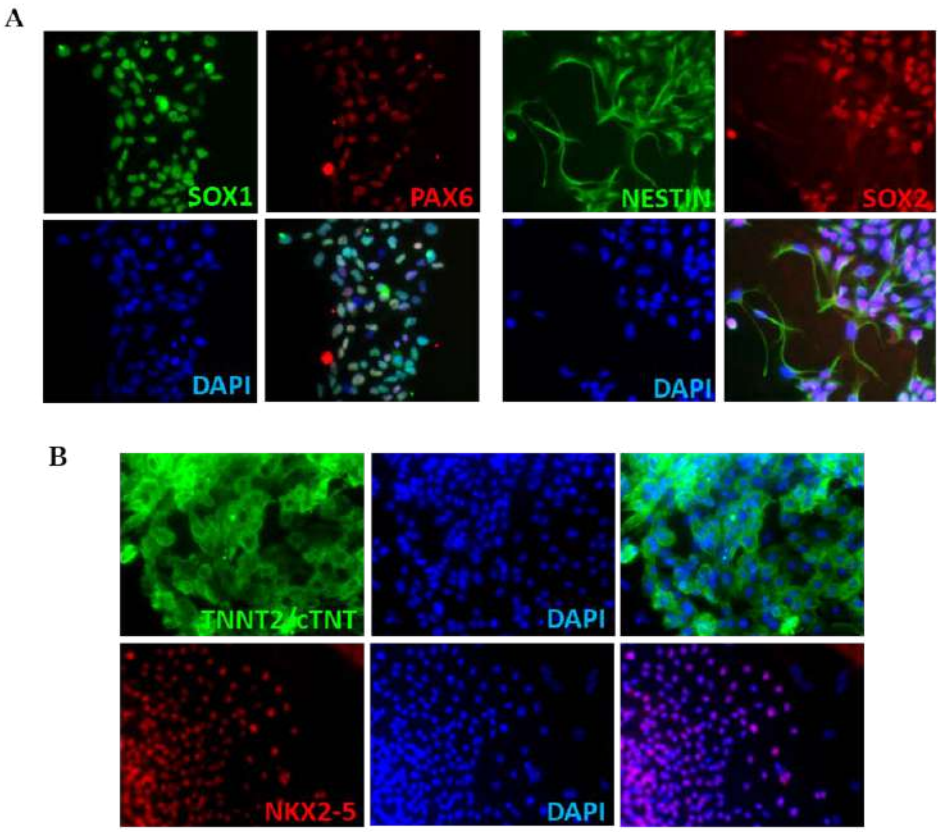

Figure 2 Analysis of tissue-specific phenotype of cells derived from iPSCs

A. Immunofluorescence confirm the neuronal phenotype of differentiated stem cells. Neural stem cells (NSC) derived from iPSCs bearing m.8344A>G mutation were stained for: i) NSC markers SOX1 (green) and PAX6 (red) and nuclear DNA (DAPI) (left, original magnification 20X), or ii) NSC markers NESTIN (green) and SOX2 (red) and nuclear DNA (DAPI) (right, original magnification 20X).

B. Immunofluorescence confirm the cardiac phenotype of MS-CM (iPSC-derived cardiomyocytes bearing m.3243A>G mutation). Differentiated cardiomyocytes were stained with TNNT2/cTNT (for cardiomyocytes, green) or NKX2-5 (for early cardiac mesoderm, red), and nuclear DNA (DAPI) (original magnification 20X).

Publications

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TARGETING TRYPANOSOMASTIDS STEROL BIOSYNTHESIS AND THIOL REDOX MECHANISM KEY ENZYMES FOR LEAD DRUG DISCOVERY

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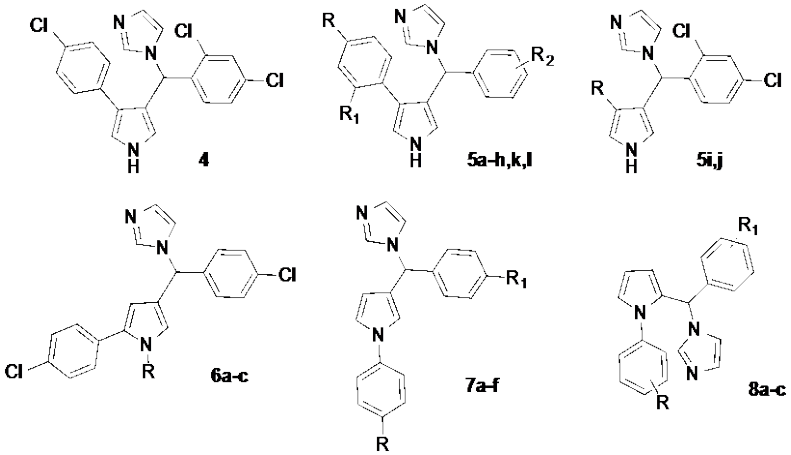
Among neglected diseases, relevant pathologies include Chagas disease, caused by *T. cruzi* and visceral leishmaniasis, caused by *Leishmania* genus, affecting nearly 20 million people worldwide. No vaccines are available and limitations of current therapy include significant toxicity, high cost and long treatment courses as well as a lack of efficacy due to the onset of drug resistance. The primary objective of this project is to develop innovative small-molecules useful as therapeutic tools for the treatment of Chagas disease and leishmaniasis. The rationale is to target and impair two central metabolic pathways unique to trypanosomatids and essential for protozoan survival: ergosterol and trypanothione pathways. This group has already identified azole derivatives endowed with very good antitrypanosomatid potencies, and, among them, derivative **4** displayed the highest anti-*T. cruzi* inhibitory potencies within the nanomolar range in phenotipic assay, proving to be effective in inhibiting TcCYP51, and in reducing parasitemia in *T. cruzi* mouse model without acute toxicity.



Thus, starting from the promising compound **4**, we designed and synthesized the new imidazoles **5**, derivatives of **4**, and compounds **6**-**8**, to perform an extensive SAR study. Furthermore, the racemic mixture of **4** was separated by HPLC on chiral stationary

phase to obtain single enantiomers (+)-**4** and (–)-**4**. All these compounds have been tested against *T. cruzi*, *L. donovani*, and *T. b. rhodesiense* and *P. falciparum*, and showed micromolar activity against *T. brucei,* low micromolar activity against *L. donovani* and nanomolar activities against *P. falciparum* (Table 1). Interestingly, the best activity was found generally against *T. cruzi* with **5i**, **6a-c** and **8b** being very potent: effective in the low nanomolar range and endowed with high selectivity indices (373 < SI < 4030). Moreover, the enantiomer (*R*)-(-)-**4** had the highest anti-*T. cruzi* activity, resulting the eutomer, effective at subnanomolar concentration (IC_{50} = 0.9 nM).

Table 1. Activity against *T. cruzi* and *P. falciparum* and cytotoxicity of selected cpds

				IC_{50}[a] (μM)				CC_{50}[b] (μM)
Cpds	R	R_1	R_2	Tc[c]	SI[d]	Pf[e]	SI[f]	
5i	2-Np[g]	-	2,4- Cl_2	0.018	363	0.34	19	6.54
6a	H	-	-	0.003	2217	0.78	9	6.65
6b	CH_3	-	-	0.003	4030	1.41	9	12.09
6c	All[h]	-	-	0.002	3880	0.63	12	7.76
8b	4-Cl	2,4-Cl_2	-	0.005	2776	0.18	77	13.88
4	Cl	H	2,4- Cl_2	0.035	275	0.16	60	9.61
(*S*)-(+)-**4**	Cl	H	2,4- Cl_2	0.017	327	0.11	51	5.56
(*R*)-(-)-**4**	Cl	H	2,4- Cl_2	0.0009	2844	0.092	28	2.56

[a]Concentration of compound required to decrease parasite viability by 50% compared to the number of parasites grown in the absence of the test compound; [b]Cytotoxicity measurement for L6 cells; [c]*Trypanosoma cruzi*, Tulahuen C2C4 amastigote; [d]Calculated as (CC_{50})/(IC_{50}); [e]*Plasmodium falciparum* K1 erythrocytic stage; [g]Np = naphtyl; [h]All= Allyl.

Effective binding of these azoles to recombinant *T. cruzi* CYP51 was demonstrated, proving that this enzyme may be targeted within *T. cruzi* parasite. An example is reported in Figure 1 for compound **6a**, that showed a K_D = 5.5 μM.

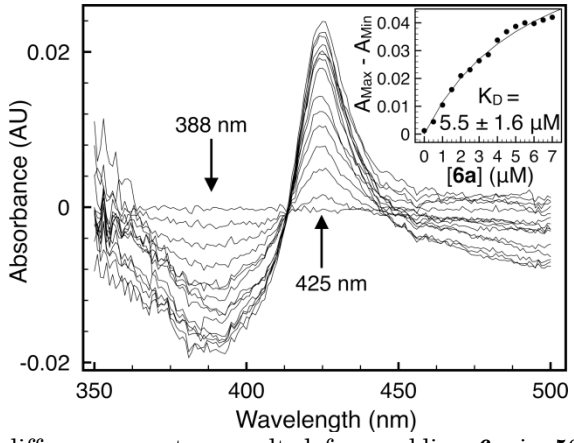


Figure 1. Type II UV-vis difference spectra resulted from adding **6a** in 500 nM increments to 2 □M TcCYP51.

Preliminary evaluation of selectivity towards relevant drug-metabolizing enzymes showed promising results, although cross-reactivity with human CYPs require further optimization for such antiprotozoal agents.
Very interstingly, **6a** and **6b** displayed remarkable efficacy in the 4-day animal model of infection resulting in >99% reduction of *T. cruzi* parasitemia in mice, with no acute toxicity observed in this animal model (Figure 2). Notably, compound **8b** was inactive in *in vivo* assays, suggesting that it is endowed with low bioavailability.
A furhter work was related to inhibitors of protozoal TR. In fact, recently, we published the cocrystal structure of TR complexed with RDS 777, a diaryl sulfide that we discovered in our laboratories. Thus, we started a structure-based anti-leishmaniasis drug design study and synthesized a series of diaryl sulfides on the basis of the structure of the above RDS 777-TR complex. The newly designed diaryl sulfides induced a dose-dependent anti-proliferative effect on *L. infantum* promastigotes. Seventy-two hours after treatment, 100 μM of each diaryl sulfide compound induced 100% promastigote mortality, whereas lower drug concentrations induced a dose-dependent anti-proliferative effect on *L. infantum* promastigotes. Table 2 reports the IC_{50} values of the tested compounds, showing that all synthesized compounds are able to kill promastigotes in micromolar range. In particular, RDS 562 showed IC_{50} = 10.79 ± 2.32 μM.

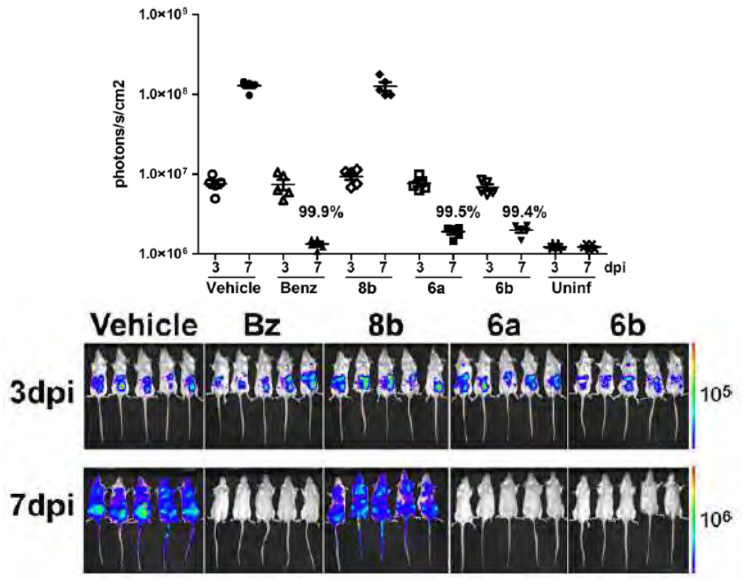


Figure 2. In vivo assays: Bz was used as reference drug; Bz, **6a** and **6b** significantly inhibited *T. cruzi* parasitemia, 99.9%, 99.5% and 99.4%, respectively. Luminescence in *T. cruzi*-infected mice measured upon luciferin injection 3 days post-infection (dpi) prior to treatment and 7 dpi after four days of treatment.

Table 2. IC_{50} values for tested compounds vs. *L. infantum* promastigotes.

Compound	IC_{50}
RDS 562	10.79 ± 2.32 μM
RDS 615	30.60 ± 1.66 μM
RDS 802	17.74 ± 1.27 μM
RDS 832	15.11 ±1.22 μM
RDS 939	16.66± 1.36 μM
RDS 1256	6.7 ± 0.21 μM

The promastigote cells treated with IC_{50} dose of all drugs (Table 2) for 24 h showed significant changes in the levels of intracellular $T(SH)_2$. A decrease of 33.15% of $T(SH)_2$ was observed in RDS 562- and of 38.5% in RDS 777-treated cells (using a three-times higher inhibitor concentration), as compared to untreated control cells (Figure 3).

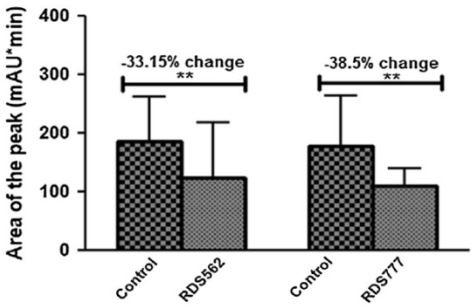


Figure 3. Percentage of trypanothione decrease in the promastigotes at a concentration equal to the IC_{50} values for the compounds RDS 777 and RDS 562.

Kinetic studies were performed on RDS 562 endowed with the highest activity against the promastigote forms of *L. donovani* and vs. trypanothione levels. RDS 562 competitively inhibited the binding of TS_2 to TR. The K_M and k_{cat} of TR used for the K_i calculation were 23.0 ± 1.0 μM and 11.4 ± 0.3 s^{-1} respectively. The value of Ki calculated from the Dixon plot analysis was 8.0 ± 1.0 μM, higher than that of Sb(III) (1.5 μM). The compound was tested also on human glutathion reductase, the human homolog of TR, and was found not effective at concentrations 10 μM and 25 μM on.

The docking studies showed that, whereas RDS 777 binds to four different TR sites (RX structure), RDS 562 is able to bind preferentially to only one of that sites in the trypanothione binding cavity whereas its affinity for the other binding sites is negligible (Figure 4).

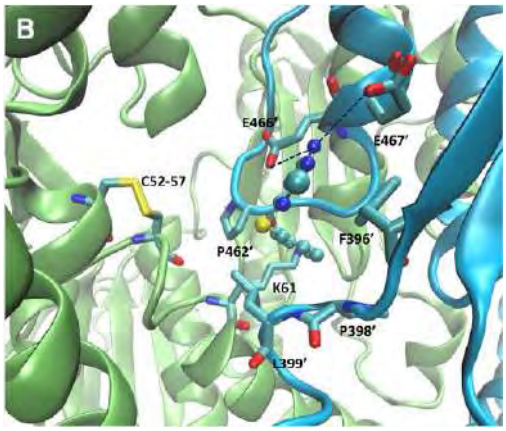


Figure 4. Binding mode of RDS 562 within the TR catalytic site.

Publications

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ONCOGENIC ROLE OF THE MHC CLASS I AMINOPEPTIDASE ERAP1 IN HEDGEHOG-DEPENDENT CANCER

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The Hedgehog (Hh) pathway is essential during embryonic development and its aberrant activation leads to tumorigenesis. Hh signalling is controlled by extracellular ligands (Hh ligands) and two transmembrane receptors (Pathched, with inhibitory function and Smoothened, acting as activator). The presence of ligand triggers an intracellular signalling cascade that removes the inhibitory effect that Patched (PTCH) exerts on the co-receptor Smoothened (SMO) and culminates with the activation of zinc-finger transcription factors of the GLI family, which in turn induces the expression of genes involved in the most important biological processes (i.e. proliferation, survival, differentiation, stemness). Because of its crucial role in cancer, the regulation of the Hh pathway and the identification of new Hh modulators have emerged as a field of great interest in tumor biology.
To address this aim, we elucidated the molecular mechanisms that involve key components of Hh signalling and whose deregulations are associated to aberrant pathway activation, leading to tumorigenesis.
The endoplasmic reticulum aminopeptidase 1 (ERAP1) is known and well studied for its immunological functions: it has been described to shape the peptide repertoire presented by MHC class I molecules, playing a role in immunity and inflammation. The role of ERAP1 in tumorigenesis is still controversial: while its loss is a frequent event and it is associated with the lack of detectable MHC class I surface expression, potentially contributing to tumor immunoescape, analysis of ERAP1 expression in human neoplastic lesions has revealed that the enzyme can be lost, acquired or retained as compared to the normal counterparts, depending on the tumor type (Fruci et al., 2008).
We discovered the oncogenic role of ERAP1, indeed we demonstrate that ERAP1 acts as positive regulator of the Hh pathway by sequestering the deubiquitylase enzyme USP47 and promoting ubiquitylation and degradation of βTrCP. Of note, all GLI transcription factors undergo ubiquitylation processes and deregulation of these events results in uncontrolled cell proliferation and tumorigenesis (Huntzicker et al., 2006). The $SCF^{\beta TrCP}$-ubiquitin ligase complex is a RING E3-ubiquitin ligase important in the regulation of GLI factors. Indeed, βTrCP degradation leads to accumulation of GLI1 and GLI2 protein levels and a reduction of the GLI3R form, thus activating the Hh pathway and stimulating cell proliferation and tumorigenesis. This study reveals an unexpected

function of ERAP1 in cancer development suggesting that targeting this aminopeptidase could open innovative perspectives for effective therapeutic approaches in the treatment of Hh-dependent tumors (**Figure 1**).

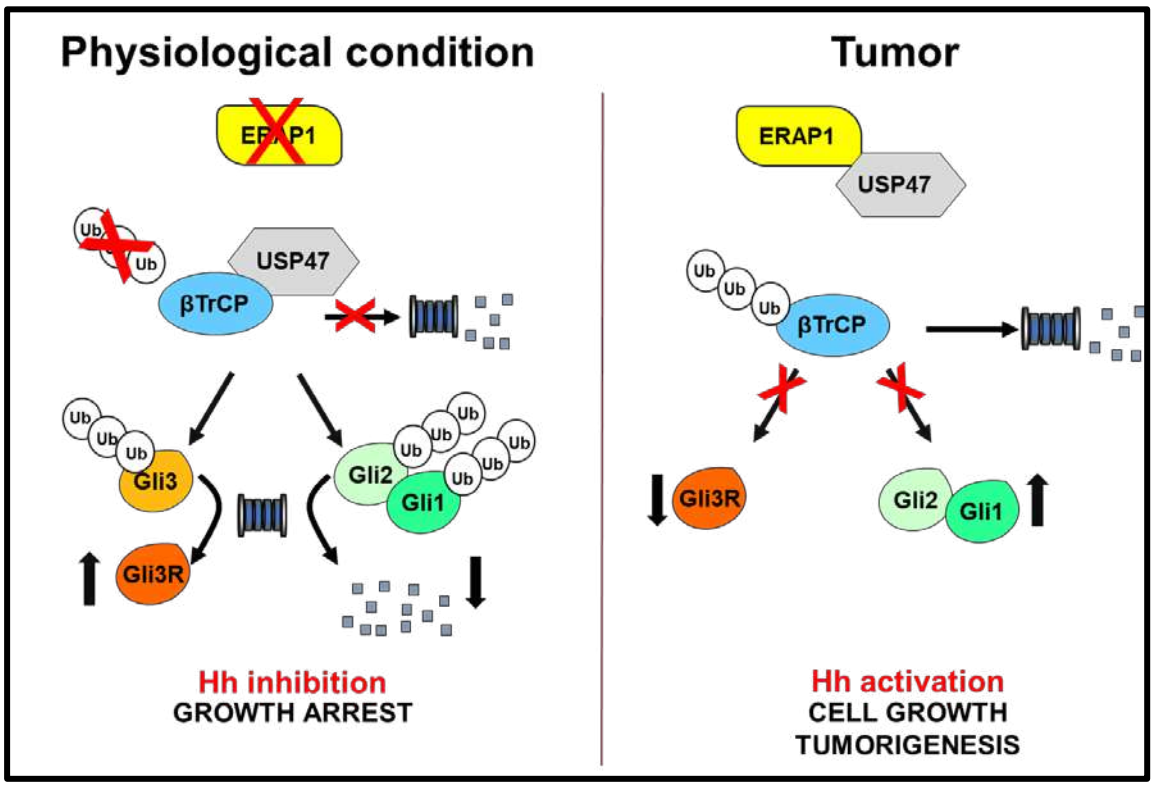

Figure 1. *Model showing the role of ERAP1 in Hh-dependent tumorigenesis.* ERAP1 promotes ubiquitylation and proteasomal degradation of βTrCP by sequestering USP47. This event leads to increase of GLI1 and GLI2 protein levels and decrease of GLI3R, thus triggering the Hh pathway and favouring cell growth and tumorigenesis. In the absence of ERAP1, USP47 binds and stabilizes βTrCP, which, in turn, promotes ubiquitylation and proteasomal degradation of GLI1 and GLI2, and ubiquitylation and proteolytic cleavage of GLI3 into the repressor form GLI3R. These events lead to the repression of the Hh pathway and inhibition of cell proliferation and tumor growth.

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"UNDER 45" RESEARCH PROJETCS

2 YEARS PROJECTS LED BY UNDER 45 YEAR OLD RESEARCHERS – CALL 2018

SECOND YEAR REPORTS

SEARCH FOR IMMUNE-RELATED PATHOGENETIC MECHANISMS OF AORTIC ANEURYSM, INDUCED BY DISRUPTED TGFB SIGNALING IN SMOOTH MUSCLE CELLS, TO FIND NOVEL THERAPEUTIC TARGETS.

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Aneurysms are focal dilatations in the wall of an artery, which are often asymptomatic until dissection or rupture occurs (Milewicz & Ramirez 2019). Thoracic aortic aneurysms (TAA) have a strong genetic basis, and often develop in individuals affected by hereditary connective tissue disorders (Milewicz & Ramirez 2019). Very few pharmacological therapies exist, and the only proven treatment option to prevent rupture is surgical repair.

Studies in the last decade have considered TGF-ß activation as the mechanism responsible for aneurysm development in different diseases (Gillis et al, 2013). Involvement of TGF-ß signaling was initially established in patients with Marfan syndrome (MFS) and in a mouse model carrying a mutation frequently found in this disease (Habashi et al, 2006). The role of TGF-ß in the development of TAA was also investigated in patients with mutations of various genes of the TGF-ß pathway (TGFBR1 and TGFBR2) in the context of different variants of Loeys-Dietz syndrome (LDS), a disorder with significant phenotype overlap with MFS. However, the signaling of TGF ß is complex, requiring a multifaceted approach to be investigated. Binding of TGF-ß to tetrameric receptor complexes composed of 2 type I (TßRI) and 2 type II (TßRII) subunits, induces receptor-mediated phosphorylation of intracellular signaling mediators Smad2 and Smad3 (mothers against decapentaplegic homolog 2 and 3) at the C-terminal Ser-X-Ser motif; binding of phosphorylated Smad2 and Smad3 (p-Smad2/3) to Smad4 induces translocation of this complex to the nucleus, and transcription of TGF-ß target genes in concert with other transcription and chromatin remodeling factors. In addition, binding of TGF-ß to its receptors can also activate Smad- independent pathways, with temporal dynamics that may vary depending on cell type.

In order to gain insights in the complex role played by TGF-ß signaling pathway in TAA, we have developed a mouse model with selective postnatal inactivation of Smad4 in Smooth Muscle Cells (SMC) (generating *Smad4*-SMC^{iko} mice), allowing to reproduce a condition of defective TGF-ß signaling in adulthood. We have reported that *Smad4*-SMC^{iko} mice develop a spontaneous aortic pathology after 45 days of TGF-ß signaling inactivation in SMC, manifested as aortic dilation and aneurysm formation (Da Ros, 2017). During 16 weeks, mice showed severe TAA and increased mortality for aortic

ruptures. At the examination of aortic tissue, a significant infiltration of innate immune cells was found, suggesting that requirement of TGF-ß in SMC to balance homeostasis and inflammation formation (Da Ros, 2017). Interestingly, histological analysis revealed progressive fragmentation of elastic lamellae with increased cellularity at sites of breaks in *Smad4*-SMC^{iko} mice as compared to control mice. In addition, mutant mice also showed alterations at the media-adventitia border of the aorta, manifested as proliferation of adventitial fibroblasts and infiltration of CD45^{+} cells, localized especially in regions of severe fragmentation of elastic lamellae. Inflammatory infiltrates at sites of disarrangement of aortic wall structure were rich in macrophages positive for CD11b and CD68; on the contrary, the presence of CD3^{+} lymphocytes was negligible. The infiltrate of immune cells in the aortic walls was better characterized by flow cytometric analysis of aorta single cells suspension. In particular, we have found a significant infiltrate of CD11b^{+} pro-inflammatory monocytes and macrophages.
Although several cytokines were found to be altered by the inactivation of Smad4, IL1-ß and Ccl2 were the dominant pathways implicated. Interestingly we found that the inactivation of Smad4 in SMCs led to an early up-regulation of IL-1ß in a cell-autonomous way. Then in turn, the production of IL-1ß activated the Ccl2-CCR2 chemokine axis for later recruited innate immune response, thus suggesting that a more complex non-cell autonomous immune mechanism is required to make the pathology progress over time.

Research aims

The project proposed specific aims to unravel the role of resident versus recruited innate immune system in the aortic pathology of *Smad4*-SMC^{iko} and to characterize novel molecular mechanisms targetable for innovative therapies. Specific aims of this project were: to investigate the non-cell autonomous determinants involved in the TAA provoked by inactivation of TGF-ß signaling in SMC, by characterizing the contribution of circulating immune system vs secondary lymphoid organs and determining the role of resident immune cells in the aorta.

Results

Although the existing paradigm of macrophage origins in vascular disease has always emphasized recruitment of bone marrow or splenic derived monocytes via the circulation, more recent data suggest that in some conditions, like the atheroma, macrophage activation and burden may be maintained predominantly by local proliferation (Robbins et al, 2013). Thus, we have performed a set of experiments aimed at investigating various routes of innate immune cells recruitment/activation in the aortic walls.
At the flow cytometry analysis of innate immune cells infiltrating the aortic walls of mice with Smad4 inactivation in SMC, as compared to control mice, we found a significant increase of macrophages and monocytes.
The increased number of infiltrating macrophages could be due to proliferation of cells in the aortic walls or to continuous recruitment from circulation or immune reservoirs.

We have previously found that IL-1ß is upregulated before infiltration of monocytes and macrophages, suggesting a role of this cytokine in transferring signals from the aortic walls to the immune system, which is activated in a second stage. By generating a further murine model where the ablation of *Smad4* in SMCs, we have found that mice with *Ccr2* deficiency (*Smad4*-SMC^{iko};*Ccr2*$^{-/-}$) were partially protected from the progression of aortic aneurysm severity, although infiltrating macrophages were still present in the aortic walls. Thus, even though a role of recruited immunity was highlighted, we could not rule out a further role of resident macrophages.
Here we have evaluated the potential contribution of various reservoirs of innate immune cells, able to respond to the Ccl2/CCR2 axis. The current paradigm of immune system activation in vascular diseases strongly points to a contribution of splenic-derived monocytes that would account for the macrophage burden in vascular tissues upon pathological conditions (Swirski et al, 2009). So far, there is no evidence of a role of this reservoir in aortic aneurysm. To evaluate the contribution of spleen versus circulating and resident macrophages to the process of immune system activation in the aortic walls of aneurysm, we have splenectomized mice before inducing *Smad4* deletion with tamoxifen (or vehicle in the control group). Then, by serial echographic analysis, we monitored the development and the progression of aneurysm, finding that no difference was determined by the absence of the spleen. In fact, both splenectomized and sham mice, after inactivation of Smad4 in SMC, showed the same timeline of aortic dilation and aneurysm progression, thus ruling out the role of this immune reservoir in the innate immune activation infiltrating the aortic walls.
The pool of monocyte/macrophages recruited by the Ccl2/CCR2 axis could also be dependent from the circulating monocytes. Thus, we performed an additional experiment by intravenously administrating clodronate liposomes or pbs liposomes as control to mice with Smad4 inactivation in SMC. At the early histological analysis, we found that although blood monocytes were depleted, aortic macrophages were still present in the aorta, thus indicating that arterial macrophages residing within the vessel may be directly activated by the loss of TGF-ß signaling. Moreover, we also found that, despite the depletion of circulating monocytes and macrophages, IL-1ß activation in SMC was still present after Smad4 inactivation. Aneurysm progression was analyzed after depletion of macrophages with clodronate liposomes. Taken together these data were suggestive of the existence of further mechanisms of immune activation in the aortic walls of *Smad4*-SMC^{iko} mice.
It has been typically recognized that macrophages in tissues like the aorta arise from transformation of Ly6C^{hi} recruited pro-inflammatory monocytes or direct recruitment of Ly6C^{low} reparative macrophages. While the recruitment of the first population usually relies on the Ccl2/Ccr2 axis that we have already tested, the second wave of Ly6C^{low} monocytes depends on a mechanism activated by CX3CR1, which is a receptor for fractalkine (Nahrendorf et al, 2007). In order to test the contribution of the latter one, the model of Smad4 deletion in SMCs was backcrossed on a background null for CX_3CR1 receptor (*Smad4*-SMC^{iko};*$CX_3CR1^{-/-}$* mice). We monitored the progression of aneurysm by

ultrasound in vivo imaging, finding no difference in the aortic dilation and vascular function between *Smad4*-SMC^{iko} mice and *Smad4*-SMC^{iko};*CX$_3$CR1*$^{-/-}$ mice. Flow cytometry analysis of single cells suspension of the aortic tissue showed that CD11b^{+} Ly6C^{lo} macrophages accumulated in the aorta of *Smad4*-SMC^{iko} mice but not in the aorta of *Smad4*-SMC^{iko};*CX$_3$CR1*$^{-/-}$ mice. These data suggest that the lack of accumulation of Ly6C^{lo} macrophages recruited by CX3CR1 doesn't affect the aortic dilation.

In order to gain information in the potential contribution of systemic and residing macrophages, the model of Smad4 deletion in SMCs was backcrossed on a background null for the orphan nuclear hormone receptor, nuclear receptor subfamily 4, group a, member 1 (Nr4a1) (*Smad4*-SMC^{iko};*Nr4a1*$^{-/-}$ mice). The Nr4a1 is essential for the tissue differentiation of recruited Ly6C^{hi} CCR2^{+} pro-inflammatory monocytes in reparative macrophages that promote the healing of the tissue (Hilgendorf et al., 2014). Interestingly, when we monitored the aortic pathology by ultrasound in vivo imaging, we found that, despite an onset of aortic dilation similar to that observe in control *Smad4*-SMC^{iko} mice, at later stages of pathology progression (12 weeks post Smad4 inactivation) *Smad4*-SMC^{iko};*Nr4a1* $^{-/-}$ mice showed a significantly increased diameter of aorta compared to *Smad4*-SMC^{iko}. Moreover, the flow cytometry analysis revealed a huge infiltrate of recruited Ly6C^{hi} CCR2^{+} pro-inflammatory monocytes in both *Smad4*-SMC^{iko} and *Smad4*-SMC^{iko};*Nr4a1* $^{-/-}$ mice. More interesting, we found an accumulation of CD11b^{+} Ly6C^{lo} macrophages in the aorta of *Smad4*-SMC^{iko} mice but not in the aorta of *Smad4*-SMC^{iko};*Nr4a1* $^{-/-}$ mice. I

Taken together these data indicate that the lack of Nr4a1 aggravates the aortic dilation, potentially promoting the inflammatory phase mediated by Ly6C^{hi} CCR2+ monocytes, being unable to convert these cells into reparative Ly6C^{lo} monocytes-macrophages. Moreover, the decreased number of CD11b^{+} Ly6C^{lo} macrophages, that represent a tissue-reparative response to counterbalance the pro-inflammatory one, results in disruption and aneurism enlargement.

In conclusion, our results propose that the signaling mediated by SMAD4 in the SMCs is necessary for the maintenance of normal aortic structure and function and that Nr4a1 is essential to limit inflammation in the aortic wall during the aneurysm formation. In fact, in the absence of Nr4a1, Ly6C^{hi} monocytes, recruited in an initial phase by the IL-1b/Ccl2 axis through the CCR2 receptor, infiltrated the aortic wall of *Smad4*-SMC^{iko} provoking a worsening of aneurysm.

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DEVELOPMENT OF A COMBINATION STRATEGY BASED ON ER AND OXIDATIVE STRESS IN ACUTE MYELOID LEUKEMIA

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AML is a very heterogeneous disease caused by different genetic aberrations. These result in the expression of fusion or mutant proteins that in all cases cause impaired differentiation and enhanced proliferation and survival of hematopoietic progenitors. At present, the only highly effective molecular targeted therapy for AML is based on all-trans retinoic acid (RA) and chemotherapy and/or arsenic trioxide (ATO) in acute promyelocytic leukemia (APL), which express the promyelocytic leukemia (PML)–retinoic acid receptor α (RARα) fusion protein. We have previously shown that APL cell lines and primary leukemic blasts induced to differentiate by RA become highly sensitive to small amounts of ER stress inducing drugs, not detrimental for the same cells in the absence of RA. Furthermore, the same cells resulted even more sensitive to a combination of RA, ER stress inducers and ATO because of generation of oxidative stress. Importantly the same treatments resulted not toxic on hematopoietic progenitors obtained from healthy bone marrows. We also observed that ER stress in combination with RA caused increased amounts of disulphide-bound high molecular weight aggregates of PML-RARα and PML, exacerbating the alteration of cellular proteostasis already generated by induction of ER and oxidative stress. This observation provided the rationale to translate the findings we obtained in APL to other types of AML characterized by fusion or mutant proteins. The presence of mutant proteins that are easily prone to aggregation or mis-folding, because of their mutant structure or because of mis-localization, could render the cells sensitive to levels of ER and oxidative stress that could be recovered in their absence.

In this project, we evaluated the impact of ER and oxidative stress inducers, used at low doses in combination with RA, in AML cell lines and primary human blasts as synergistic strategy to promote AML cell death. We demonstrated that the triple combination of the differentiating agent retinoic acid (RA), the ER stress-inducing drug Tunicamycin (Tm) and arsenic trioxide (ATO), able to generate oxidative stress, leads to death of AML cell lines expressing fusion proteins involving the gene MLL and the internal tandem duplication (ITD) in the FLT3 tyrosine kinase receptor (Figure 1A). Importantly, the combination of RA, Tm and ATO decreased the colony forming capacity of primary leukemic blasts bearing the mutation FLT-ITD, but not those carrying other mutations, without affecting healthy hematopoietic progenitor cells (Figure 1B and C).

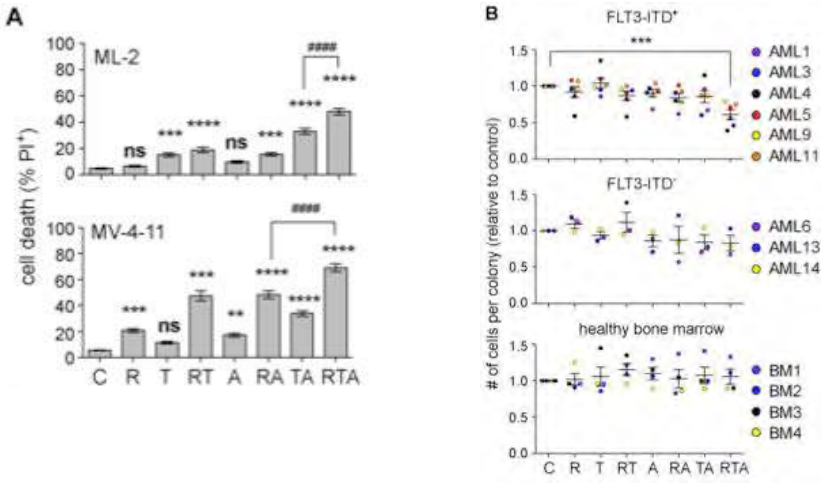
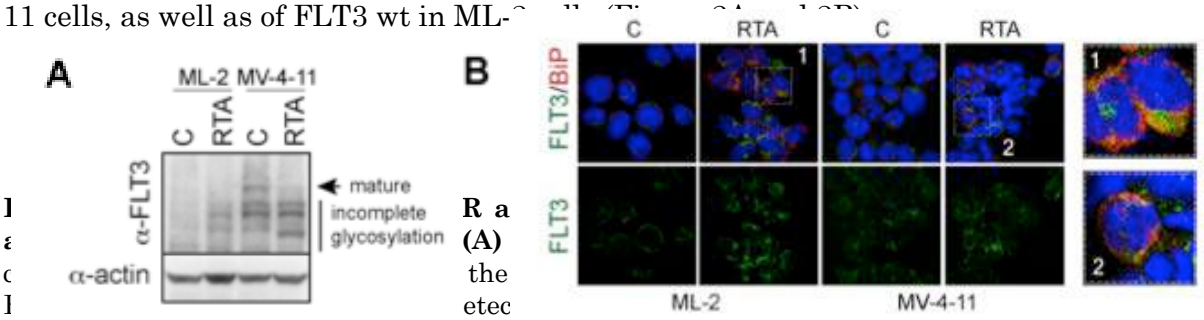


Figure 1- The combination of ER and oxidative stress with RA induces AML cell death. **(A)** ML-2 and MV-4-11 cells, treated for 72 hours with 10nM RA, 50ng/ml Tm and 500nM ATO alone or in combination, respectively indicated by R, T and A, were analyzed for propidium iodide (PI) uptake to evaluate cell death (n=16 ± SEM). C indicates control, vehicle-treated cells. **(B)** Colony forming unit assay for AML blasts isolated from the bone marrow of six patients FLT3-ITD positive, three FLT-ITD negative and for mononucleated cells isolated from four healthy donors. The cells were treated in semisolid medium with 10nM RA, 50ng/ml Tm and 500nM ATO alone or in combination as indicated. After 8 days, the colony size (number of cells/colony) was evaluated by microscopy. The graphs report the ratio of the average number of cells forming the colonies of each treated sample over its control (***Student's T test p value < 0.002).

We also showed, in cell lines, that combination of these drugs impairs maturation and causes accumulation of FLT3 protein in the ER and generates ER and oxidative stress. It is established that the internal tandem duplication of FLT3 impairs its full glycosylation and folding causing its retention in the ER. Accordingly, we observed that FLT3-ITD is partially retained in the ER in an immature form and that treatment with RTA caused further hindrance of protein maturation and retention in the ER of FLT3-ITD in MV-4-11 cells, as well as of FLT3 wt in ML-



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immature, not fully glycosylated forms upon treatment with RTA. MV-4-11 cells carry a homozygous FLT3-ITD mutation that is mostly retained intracellularly in immature forms (C). Treatment of cells with RTA further impaired FLT3-ITD glycosylation as demonstrated by the disappearance of the fully glycosylated (mature) form and by the increase of the less glycosylated form. **(B)** Confocal analysis of the distribution of FLT3 and BiP proteins in ML-2 and MV-4-11 cells in control and RTA-treated cells. According to the observations obtained by western blot analysis in (A) FLT3 is found on plasma membrane of ML-2 control cells and accumulates in the ER upon treatment with RTA. BiP is the main ER chaperone and co-localization of FLT3 with BiP demonstrates misfolding and retention in the ER (yellow spots in inset 1). FLT3-ITD is already retained intracellularly in control MV-4-11 cells and treatment with RTA increases the amount of FLT3-ITD in the ER (inset 2).

Tm activated the UPR in ML-2 and MV-4-11 cells either alone or in combination with RA and/or ATO (Figure 3A). Treatment with ATO alone triggered the oxidative stress response, that reached much higher levels upon ATO in combination with RA and Tm, as shown by increased expression of the gene HMOX-1, a main player of this response (Figure 3A). ER stress and oxidative stress are tightly linked even though the mechanisms of interaction between these stresses are still unknown. Indeed, generation of reactive oxygen species and consequent oxidative stress is one of the mechanisms triggered by the unfolded protein response to induce apoptosis in the presence of overwhelming ER stress, and on the other hand altered redox homeostasis induces ER stress. Our data suggest that the ER stress response triggered by Tm alone and the oxidative stress response triggered by ATO alone re-establish homeostasis resulting in cell survival whereas the combination of Tm with ATO and RA leads to overcoming levels of oxidative stress and consequent cell death. Indeed, the antioxidant agent N-acetylcysteine (NAC) rescued the deleterious effects of the combinations TA and RTA on cell viability of ML-2 cells (Figure 3B) and reduced oxidative and ER stress (Figure 3B and C). The chemical chaperone sodium-4-phenylbutyrate (PBA), that favors protein folding, produced similar, although less marked effects than NAC (Figure 3B and C).

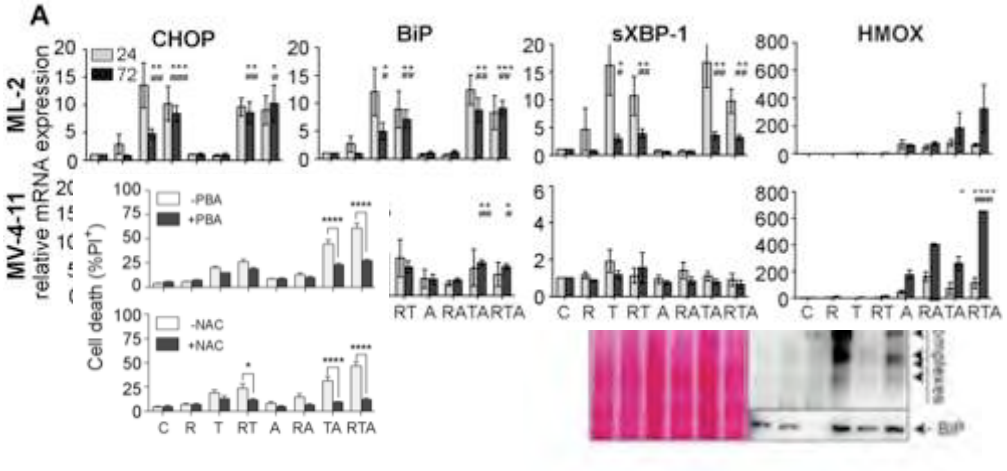

Figure 3- Treatment of ML-2 and MV-4-11 cells with the combination RTA triggers the UPR and the oxidative stress response. (A) The RNA of ML-2 and MV-4-11 cells, treated as described in Figure 1A for 24 or 72 hours, was analyzed by qRT-PCR for the expression of UPR target genes **(B)** ML-2 cells, treated for 72 hours as in Figure 1A in the presence or not of 20mM N-acetylcysteine (NAC) or of 2.5mM sodium-4-phenylbutyrate (PBA), were analyzed for propidium iodide (PI) uptake to evaluate cell death (NAC n=4 ± SEM; PBA n=3 ± SEM). **(C)** Western blot of protein extracts from ML-2 cells, treated as in (A) to detect the complexes among the chaperone BiP and its client misfolded proteins.

At present our data provide a proof of concept that low amounts of drugs that generate ER and oxidative stress combined with RA could be an effective targeted therapy to hit AML cells characterized by MLL fusion proteins and FLT3-ITD mutation. Identification of new molecular targets like the oxidative and the ER stress responses would allow the design of new sub-type related therapeutic strategies.

Publications

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FUNCTIONAL CHARACTERIZATION AND PHARMACOLOGICAL INHIBITION OF COLISTIN RESISTANCE IN *PSEUDOMONAS AERUGINOSA*

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The world is currently facing a worrying threat due to the emergence of multidrug resistance in many Gram-negative bacterial pathogens. The lack of new antimicrobials active against these bacteria has prompted to re-evaluate the use of the old polymyxin antibiotic colistin (polymyxin E) as a last-resort option to treat recalcitrant Gram-negative infections.
Colistin is a cationic polypeptide, and its antibacterial activity relies on its interaction with the negatively charged lipid A moiety of lipopolysaccharide (LPS) in the outer membrane of Gram-negative bacteria. This leads to the displacement of Ca^{2+} and Mg^{2+} cations that stabilize the LPS layer, causing derangement of the outer membrane, increased membrane permeability, leakage of cell contents, and ultimately cell death (Nation & Li 2009, doi:10.1097/QCO.0b013e328332e672).
Reintroduction of colistin in clinical practice has inevitably led to the emergence of colistin-resistant isolates (Jeannot *et al.* 2017, doi: 10.1016/j.ijantimicag.2016.11.029). Gram-negative bacteria acquire resistance to colistin primarily through genomic mutations in regulatory genes causing transcriptional activation of genes responsible for remodeling of LPS, through the covalent addition of 4-amino-4-deoxy-L-arabinose (L-Ara4N) or phosphoethanolamine (PEtN) to the lipid A moiety. These modifications reduce the negative charge of LPS and, therefore, its affinity for colistin, ultimately leading to resistance (Olaitan *et al.* 2014, doi: 10.3389/fmicb.2014.00643).
The present project was aimed at assessing the relevance of the L-Ara4N modification for colistin resistance, as well as at investigating its suitability as molecular target for the development of colistin resistance inhibitors, using the opportunistic human pathogen *Pseudomonas aeruginosa* as model organism. *P. aeruginosa* is responsible for severe infections in immunocompromised patients and represents a major threat to patients suffering from cystic fibrosis (CF), in whom *P. aeruginosa* frequently causes life-threatening chronic lung infections. Colistin is extensively used for treating multidrug-resistant *P. aeruginosa* infections, especially in CF patients. Unavoidably, colistin-resistant *P. aeruginosa* strains are frequently isolated in CF and non-CF centers (Falegas *et al.* 2010, doi: 10.1016/j.drup.2010.05.002).
In *P. aeruginosa* colistin resistance is generally associated with overexpression of the *arn* operon, encoding the enzymes for L-Ara4N modification of lipid A. The expression of this operon is controlled by a complex regulatory network involving at least five two-component systems (TCSs). Mutations within these TCSs that result in the constitutive activation of the *arn* operon are typically identified in colistin-resistant *P. aeruginosa* (Jeannot *et al.* 2017, doi: 10.1016/j.ijantimicag.2016.11.029; Olaitan *et al.* 2014, doi: 10.3389/fmicb.2014.00643; Falegas *et al.* 2010, doi: 10.1016/j.drup.2010.05.002). However, some *in vitro* studies provided evidence that individual TCSs are not essential

for the acquisition of colistin resistance in *P. aeruginosa*, leading to the hypothesis that alternative or compensatory mechanisms may exist (Lee *et al.* 2014. doi.org/10.1093/jac/dku238).

The first aim of the project was to investigate whether lipid A aminoarabinosylation is required and/or sufficient for colistin resistance acquisition in *P. aeruginosa.* To this aim, in the first year of activity we generated (i) mutant strains unable to synthesis L-Ara4N (Δ*arnBCA* mutants) and (ii) recombinant strains which constitutively express the *arn* genes (P*rpsA*::*arn* strains, in which the *arn* promoter has been replaced with the promoter of the housekeeping gene *rpsA*). These mutant and recombinant strains were generated both in reference laboratory strains (PAO1 and PA14) and in a small collection of clinical isolates from CF and non-CF patients. To assess whether L-Ara4N-independent colistin resistance mechanisms exist in this bacterium, Δ*arnBCA* mutants were compared to parental strains in long term experimental evolution assays, in which the bacteria were sequentially cultured in the presence of increasing concentrations of colistin in the attempt to select for mutants that acquire successive mutations leading to high-level colistin resistance. In several independent assays, the Δ*arnBCA* mutants of both reference and clinical *P. aeruginosa* strains never grew with colistin concentrations ≥ 4 μg/ml, which corresponds to the epidemiological cutoff (ECOFF) of colistin for *P. aeruginosa*, *i.e.* the highest MIC for isolates devoid of any detectable acquired resistance mechanisms (Ellington *et al.* 2017. doi.org/10:1016/j.cmi.2016.11.012). In contrast, the parental strains always acquired the ability to grow in the presence of high colistin concentrations, and these resistant mutants showed very high colistin MIC (≥ 64 μg/ml). Overall, this evidence directly confirmed that lipid A aminoarabinosylation is an essential prerequisite for the development of colistin resistance in *P. aeruginosa* (Lo Sciuto & Imperi 2018, doi: 10.1128/AAC.01820-17). In addition, we also started to investigate whether lipid A aminoarabinosylation is sufficient to confer colistin resistance to *P. aeruginosa.* To this aim, we compared *arn* gene expression, lipid A aminoarabinosylation levels and colistin MIC between parental and recombinant P*rpsA*::*arn* strains (see above). qRT-PCR and mass spectrometry assays confirmed that the replacement of the *arn* promoter with the constitutive *rpsA* promoter leads to *arn* gene overexpression and modification of lipid A with L-Ara4N. Notably, lipid A aminoarabinosylation increased colistin resistance in most but not all strains, as some recombinant P*rpsA*::*arn* strains showed minimum inhibitory concentration (MIC) of colistin comparable to parental strains. Moreover, we observed that the increase in colistin resistance in P*rpsA*::*arn* strains is culture condition-dependent, being much higher in media containing high concentrations of divalent cations and under conditions mimicking *P. aeruginosa* infections, such as growth in human serum and in artificial CF sputum. Overall, these analyses revealed that the degree of colistin resistance conferred by L-Ara4N is strain- and culture condition-dependent, and that the evolution of high colistin resistance levels in *P. aeruginosa* likely requires some genetic and/or phenotypic adaptation(s) in addition to lipid A aminoarabinosylation, as also suggested by other groups (Jochumsen *et al.* 2016, doi: 10.1038/ncomms13002).

In the second year of activity, we investigated the effect(s) of L-Ara4N-modified lipid A

on *P. aeruginosa* fitness, as this information is important to predict the evolution rates and the spread of colistin resistance in this bacterium. By comparing recombinant P*rpsA*::*arn* strains with their wild type counterparts in a number of *in vitro* and *in vivo* assays, we found that lipid A aminoarabinosylation does not significantly affect bacterial growth (in different media), biofilm formation, cell wall stability, and functionality of the outer membrane permeability barrier. Moreover, experiments in a simple infection model based on the larvae of the insect *Galleria mellonella* did not reveal any detrimental effect of lipid A aminoarabinosylation on *P. aeruginosa* infectivity (Lo Sciuto *et al.*, manuscript submitted to Int J Antimicrob Agents).
In the second year we also carried out a side project to evaluate the possible contribution of another lipid A modification, *i.e.* the hydroxylation of secondary acyl chains, to colistin resistance. Indeed, lipid A hydroxylation, that is catalyzed by the dioxygenase LpxO, was found to be important in some Gram-negative bacteria for resistance to cationic antimicrobial peptides (such as colistin), and also for survival in human blood and pathogenicity in animal models (Mills *et al.* 2017, doi: 10.1128/IAI.00068-17; Bartholomew *et al.* 2019, doi: 10.1128/IAI.00066-19). Interestingly, while all LpxO-proficient bacteria investigated so far have a single hydroxylated secondary acyl chain in lipid A, the lipid A of *P. aeruginosa* can be hydroxylated in both secondary acyl chains, and two *lpxO* orthologs have been identified in *P. aeruginosa* genomes (King *et al.* 2009, doi: 10.1177/1753425909106436). However, the functionality of these genes, as well as the physiological role of lipid A hydroxylation in *P. aeruginosa*, was not investigated yet. By generating single and double deletion mutants in the *lpxO1* and *lpxO2* homologs of *P. aeruginosa* PAO1, as well as LpxO1 and LpxO2 overexpressing strains, we demonstrated that both LpxO1 and LpxO2 are responsible for lipid A hydroxylation, and that they act on different secondary acyl chains. Notably, our *in vitro* assays revealed that, in *P. aeruginosa*, lipid A hydroxylation does not affect bacterial growth, biofilm formation, cell wall stability, persistence in human blood, and resistance to colistin and other antibiotics. In contrast, it is required for full infectivity in the *G. mellonella* infection model, even if it does not influence bacterial survival within the larval hemolymph. Overall, this study led to suggest that lipid A hydroxylation may play a role in *P. aeruginosa* virulence that is likely not directly related to outer membrane integrity (Lo Sciuto *et al.* 2019, doi:10.3390/pathogens8040291).

The second aim of the project was to validate lipid A aminoarabinosylation as a pharmacological target for compounds able to restore colistin sensitivity in resistant isolates and/or to hinder the emergence of colistin resistance. Through a docking-based virtual screening for inhibitors of ArnT, *i.e.* the integral membrane enzyme responsible for L-Ara4N attachment to lipid A (Petrou *et al.* 2016, doi: 10.1126/science.aad1172), performed in collaboration with Bruno Botta (Sapienza University) and Mattia Mori (University of Siena), we had previously identified a natural compound (hereafter named BBN149) that partially reverted colistin susceptibility in a colistin-resistant *P. aeruginosa* isolate. BBN149 is a natural diterpenoid (Fig. 1) isolated from the leaves of

Fabiana densa var. *ramulosa* (Erazo *et al.* 2002. Planta Med).

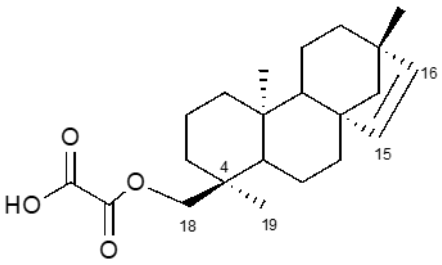

Fig. 1. Structure of the diterpenoid BBN149 (ent-beyer-15-en-18-O-oxalate).

In the first year of activity, we demonstrated that BBN149 has a specific and wide range activity, being active against all colistin-resistant *P. aeruginosa* isolates tested so far, without influencing colistin resistance levels in susceptible strains (that do not express the *arn* operon). As expected, BBN149 had no effect on *P. aeruginosa* growth and cell viability in the absence of colistin. These results supported the hypothesis that BBN149 specifically targets colistin-resistance mechanism(s). In parallel, we demonstrated that BBN149 is not toxic to lung epithelial cells, at least at the concentrations tested in our assays, and that it is also active against a small collection of recently-characterized colistin-resistant isolates of a different Gram-negative bacterium (*Klebsiella pneumoniae*) (Esposito *et al.* 2018, doi: 10.3389/fmicb.2018.01463).

In the second year of activity, we further confirmed the specificity of BBN149 towards colistin resistance by showing that this compound does not increase the susceptibility of *P. aeruginosa* to other antibiotics with different mechanisms of action. Moreover, in order to verify whether BBN149 actually targets ArnT-mediated lipid A aminoarabinosylation, we assessed the effect of BBN149 on lipid A aminoarabinosylation levels by mass spectrometry. This analysis revealed that BBN149 slightly reduces but does not completely inhibit lipid A aminoarabinosylation in colistin-resistant *P. aeruginosa* isolates, and that the effect is quite variable among different strains. Experiments are ongoing the verify the inhibitory effect of BBN149 on lipid A aminoarabinosylation in *K. pneumoniae.* Although preliminary, the above evidence suggests that BBN149 could have other targets and/or activities besides, or alternative to, ArnT inhibition. In the next months, we aim at selecting BBN149 insensitive *P. aeruginosa* mutants in order to detect by whole genome sequencing the mutations and, thus, the genes that could be involved in protection from BBN149 activity, likely providing information about its mechanism(s) of action.

Concurrently, in collaboration with Bruno Botta, we chemically synthesized or isolated from *F. densa* leaves a number of BBN149 derivatives, that were compared to BBN149 for the capability to counteract colistin resistance in *P. aeruginosa.* Several compounds that relevantly potentiates colistin activity against a colistin resistant tester strain were identified, although none of the them was significantly more effective than BBN149. Nevertheless, this analysis allowed us to characterize the backbone structure and functional groups important for BBN149 activity. In particular, we observed that (i) the unsaturation between C15-C16 is not required for activity, (ii) the configuration of C4 (R or S) only marginally affects activity, (iii) the oxalate group at C18 (or C19) is essential for activity; (iv) the presence of a carboxylic group, an alcohol or a methyl ester at C19 and a carbonyl group at C16 completely abrogates activity (Fig. 1). This information will

be used to design and synthesize a second generation of BBN149 analogues, with the final goal to obtain compounds with higher activity and/or improved water solubility, as these represent important issues for the *in vivo* efficacy tests.

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CELLULAR PATHWAYS INVOLVED IN THE TOXICITY OF NEUROSERPIN POLYMERS THAT CAUSE THE DEMENTIA FENIB

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Serpins are the largest and most broadly distributed superfamily of protease inhibitors (Irving et al., 2000, *Genome Res*). Members of the serpin superfamily are found in all major branches of life including prokaryotes and eukaryotes, and are characterised by more than 30% amino acid sequence homology with the archetypal serpin alpha-1 antitrypsin, a secretory protein produced in the liver and delivered to the blood so it can reach the lungs to inhibit the protease neutrophil elastase (Gooptu and Lomas, 2009, *Ann Rev Biochem*). Amino acid substitutions in serpins result in pathologies collectively known as serpinopathies. These are protein conformational diseases characterised by polymerisation and intracellular deposition of mutant serpin variants within the endoplasmic reticulum (ER). The neurodegenerative pathology FENIB (familial encephalopathy with neuroserpin inclusion bodies) is an autosomal dominant serpinopathy caused by mutations in neuroserpin (NS) that cause polymer formation (Davis et al., 1999, *Nature*). Six different mutations have been described that promote NS polymerisation in FENIB patients: Ser49Pro, Ser52Arg, His338Arg, Gly392Glu, Gly392Arg and Leu47Pro. Mutant NS was found to accumulate within neurons forming periodic acid-Schiff (PAS)-positive inclusion bodies known as Collins bodies, most abundant in the cerebral cortex but also present in other regions of the central nervous system (Davis et al., 1999, *Am J Pathol*), causing symptoms that range from dementia to epilepsy (Roussel BD et al., 2016, *Epileptic Disord*). The phenotypical and biochemical features of FENIB have been deduced from its clinical manifestations and from the postmortem analysis of affected brains, and have been confirmed through the expression of mutant NS in diverse cellular systems (Miranda et al., 2004, *J Biol Chem*; Miranda et al., 2008, *Hum Mol Genet*; Roussel et al., 2013, *Hum Mol Genet*; Moriconi et al., 2015, *FEBS J*), in *Drosophila melanogaster* (Miranda et al., 2008, *Hum Mol Genet*) and in mice (Madani et al., 2003, *Mol Cell Neurosci*; Galliciotti et al., 2007, *Am J Pathol*). Transgenic mice overexpressing S49P and S52R NS showed the formation of abundant intraneuronal Collins bodies, neuronal loss in the cerebral cortex and hippocampus and pathological phenotypes reminiscent of FENIB during late adulthood. Overexpression of human S49P, S52R, H338R and G392E NS in *Drosophila melanogaster* led to a decrease in locomotor activity, with decreasing mobility correlating to increased polymer content in the brain. In cellular models, polymer formation and its correlation with the disease phenotype was confirmed in transiently transfected COS-7 and stable inducible PC12 cell models of FENIB, where overexpression of each mutant variant lead to intracellular accumulation of polymeric NS within the ER to a degree that was proportional to the severity of FENIB seen in patients. Despite these results, these cellular systems failed to show clear signs of cell malfunction and death upon NS polymer accumulation, precluding a detailed investigation of the mechanisms underlying NS polymer toxicity.

The lack of a toxic phenotype in cellular models of FENIB could be related to the proliferative nature of the cell lines used so far. To overcome this issue, we developed a neuronal model with stable overexpression of wild type (WT), G392E and delta NS, or control green fluorescent protein (GFP). Delta NS is a mutant variant with a premature stop codon causing the synthesis of truncated NS that cannot polymerise and, contrary to polymers, activates a classical unfolded protein response (UPR) (Davies et al., 2009, *J Biol Chem*). Mouse neural progenitor stem cells (NPSCs) were isolated from the mouse foetal brain cortex, stably transfected with the three NS variants described above and propagated *in vitro,* either under proliferative conditions or differentiating them to mature, non-dividing neurons. We found that in differentiated NPSCs, expression of polymerogenic NS led to the upregulation of several genes involved in the defence against oxidative stress (Guadagno et al., 2017, *Neurobiol Dis*). Oxidative stress, the imbalance between generation and disposal of reactive oxygen species (ROS), is an important factor in several neurodegenerative disorders including Alzheimer's, Parkinson's and Huntington's diseases and amyotrophic lateral sclerosis (Cobb and Cole, 2015, *Neurobiol Dis*). Neurons are particularly vulnerable to oxidative stress due to their high energy requirements, the decrease in antioxidant defences with age and their terminally differentiated nature (Gandhi and Abramov, 2012, *Oxid Med Cell Longev*). Within the ER, ROS can be generated as a by-product of physiological protein oxidation and upon ER stress due to accumulation of misfolded proteins. Serpin polymerisation may upset the redox balance in the ER, and this perturbation could affect the redox state of mitochondria through mitochondrial-ER contact sites. Mitochondria are particularly important in the nervous system, where their function is essential to neuronal processes such as energy production, Ca^{2+} regulation, maintenance of plasma membrane potential, protein folding by chaperones, axonal and dendritic transport, and the release and re-uptake of neurotransmitters at synapses, and they present a highly dynamic behaviour comprising mitochondrial fusion and fission events (Hoppins et al., 2007, *Annu Rev Biochem*; Zhang et al., 2007, *FEBS Lett*).

We thus decided to investigate the state of the mitochondrial network in our cell lines by staining them with Mitotracker Red CMXRos ® (Mitotracker), a red fluorescent dye that labels mitochondria in living cells using their membrane potential. Based on the literature and our analysis of the cellular phenotypes, we defined three categories of mitochondrial distribution: i) network: healthy cells showed mitochondria homogeneously distributed as a filamentous network throughout the cytoplasm; ii) perinuclear: mitochondria that appeared clustered in the neuronal soma, generally next to the nucleus; and iii) fragmented: mitochondria appeared as small, rounded and located close to the nucleus. In our analysis, we grouped perinuclear and fragmented

mitochondria as altered distribution. In basal conditions, GFP, WT and delta NS expressing cells mostly showed the network distribution, indicative of healthy mitochondria. Cells expressing G392E NS showed a higher proportion of altered mitochondria (nearly 40%), which were often found clustered close to the nucleus with no mitochondrial fragmentation. This toxic phenotype was due to polymer accumulation within the ER rather than to the presence of polymers in the culture medium, since no mitochondrial alterations where found in control GFP neurons treated with culture medium conditioned by G392E NS cells. We also observed that alterations in mitochondrial distribution correlated with modifications in the morphology of differentiated neurons. By double staining of mitochondria and the actin cytoskeleton (with a phalloidin-fluoresceine conjugate), neurons with perinuclear or fragmented mitochondria lacked the long neurites typical of healthy cells. This was confirmed by measuring neurite length that showed shorter neurites in G392E NS neurons when compared to WT NS ones.

To better understand these alterations in mitochondrial distribution we performed pharmacological inhibition and enhancement of the cellular defences against oxidative stress. Glutathione (GSH) is an important antioxidant metabolite that prevents cell damage caused by the presence of ROS, lipid oxidases and free radicals, and is involved in the mechanism of action of many enzymes that were overexpressed in our G392E NS expressing cells (Guadagno et al., 2017, *Neurobiol Dis*). We depleted GSH by treating our cells with DEM (diethyl maleate, 50 µM) and assessed their mitochondrial network with Mitotracker. Cells overexpressing GFP, WT and delta NS showed limited alterations in mitochondrial morphology after DEM treatment, while cells expressing G392E NS showed a significant increase in fragmented mitochondria, supporting the oxidative nature of the insult caused by polymerogenic G392E NS. We sought further evidence by investigating if antioxidant treatments could recover the perinuclear distribution seen in the G392E NS cells. We used melatonin, a pineal gland neurohormone that mediates photoperiodicity in mammals and is also a well characterised antioxidant, and alpha tocopherol, a vitamin that functions as a lipid-soluble antioxidant protecting cell membranes from oxidative damage. We first treated WT and G392E NS cells with increasing concentrations of either molecule for 24 and 48 h and stained them with Mitotracker to assess mitochondrial distribution. After determining the optimal concentration for each molecule (10 µM in both cases), we treated all cell lines with each antioxidant during the last 48 h of the differentiation treatment. All cell lines showed some improvement in mitochondrial distribution in response to both antioxidants, but for cells expressing GFP, WT and delta NS the effect was only mild, while cells

expressing G392E NS showed a clear recovery and reduction of the perinuclear phenotype to values similar to control cells.

To gain functional insight into mitochondrial alterations in cells expressing G392E NS we used the fluorescent probe JC-1, which reports on the state of the inner mitochondrial membrane potential and can be an earlier marker of mitochondrial dysfunction. We found a significant decrease in JC-1 transport into the mitochondrial matrix in G392E NS neurons, indicative of a reduced potential of their inner mitochondrial membrane. This result led us to investigate mitochondrial metabolism in our neuronal lines by measuring several parameters related to respiration in a Seahorse platform through a collaboration with Prof. Michael Duchen at UCL (London, UK). Analysis of the oxygen consumption rates under different experimental conditions showed no significant differences between the four cell lines, probably due to the mixed character of our differentiated cultures, which contain glial as well as neuronal cells, but also suggesting that mitochondrial function is not excessively altered in G392E NS neurons, in agreement with the lack of neuronal death observed for these cells under basal conditions.

The ER and mitochondria are intimately related, with the homeostasis of both organelles finely tuned by their interaction at particular regions of contact between their membranes, the mitochondria-ER sites (MAMs). Since NS polymers accumulate inside the ER, we also investigated the crosstalk between ER and mitochondria by assessing the extent of MAMs in G392E NS neurons compared to WT NS ones. Our results showed a decrease in the area of contact between the two organelles when assessed by high resolution confocal microscopy, performed in collaboration with Dr. Giovanna Galliciotti at University Medical Center Hamburg-Eppendorf (Germany). Furthermore, by western blot analysis of WT and G392E NS cells we confirmed a decrease in several membrane proteins (VDAC, Sigma1 receptor and IP3 receptor) that take part in the interaction between the ER and mitochondria at MAMs. A similar phenotype has been observed in cells expressing a mutant α-synuclein variant found in patients with familial Parkinson's disease (Cali et al., 2012, *J Biol Chem*).

Misfolded protein accumulation within the ER leads to activation of the UPR, a stress signalling pathway from the ER to the nucleus. NS polymers, contrary to most forms of protein aggregation, do not activate this pathway in the cell models studied so far. Since our system represents the first neuronal cell culture model for FENIB, we are currently investigating if NS polymers elicit the UPR in neurons or, on the contrary, polymer accumulation in the ER is toxic by a mechanism independent from classical UPR.

As part of our research on serpin polymers and using approaches that are complementary to our NS studies, we also investigate the polymerisation of the archetypical serpin alpha-1 antitrypsin (AAT), in collaboration with Prof. David Lomas and his group at UCL (London, UK). Our contribution in these studies includes the production of conformation-specific and functional monoclonal antibodies, which has recently led to the characterisation of two novel reagents: an antibody able to interfere with the inhibitory activity of AAT against several cognate proteases (elastase, chymotrypsin and proteinase 3), and an antibody that does not bind the main polymerogenic variant of AAT, which we have used to demonstrate the presence of heteropolymers formed by wild type and mutant AAT in the liver and blood of heterozygous patients of AAT deficiency.

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MOLECULAR BIOMARKERS PREDICTIVE OF HEMATOLOGICAL RESPONSE TO DIRECT ANTIVIRAL THERAPY IN HEPATITIS C VIRUS ASSOCIATED LYMPHOPROLIFERATIVE DISORDERS

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Hepatitis C virus associated lymphoproliferative disorders (LPDs) range from benign diseases, as mixed cryoglobulinemia (MC) to malignant lymphomas, usually indolent B-cell non-Hodgkin lymphomas (NHL). Dependence of the LPDs from the viral chronic infection provided by HCV has been postulated based on the regression of these diseases after virus eradication, but the inciting viral antigen, responsible for B cell stimulation, has not yet been identified. The advent of direct acting antivirals (DAA) able to eradicate HCV in less than one month has completely changed the natural history of HCV-associated LPDs and it is likely that in a not too far future this infection will be eliminated. Now we assist to a widespread virus eradication associated with high efficacy in the cure of extrahepatic manifestations as MC and NHLs. Nonetheless both situation seem not to be completely defeated as relapses of MC vasculitis and partial or non-response of NHL have been described by other and our group (Visentini M. Rheumatology 2018; Arcaini L. et al. Blood 2016, Frigeni M et al. Leukemia 2019).

This project has the aim to identify molecular and cellular characteristics of B cells clonally expanded in MC and in NHL able to predict the response to virus eradication and/or to shed light in the pathogenic mechanisms involved in disease relapse and non-response.

We investigated IGHV-D-J rearrangements and IGKV-J/IGLV-J rearrangements of 13 patients with HCV-related LPDs in an attempt to untangle the relative role of immunoglobulin heavy and light chains in generating potentially autoreactive or virus-specific B cell receptors (BCRs). We then correlated signatures of BCR stereotypy and homology with RFs or anti-HCV E2 antibodies with the response of LPDs to antiviral therapy.

While only two patients shared a stereotyped heavy chain complementarity determining region 3 (CDR3) sequence, two kappa chain CDR3 stereotyped sequences accounted for 77% of BCRs. Light chains were enriched in sequences homologous to anti-HCV E2 antibodies compared to heavy chains (7/13 vs 0/13; p=0.005). Anti-HCV E2 homology was uniquely associated (7/7 vs 0/6; p=0.0006) with a stereotyped CDR3 sequence encoded by IGKV3-20/3D-20 gene(s) accounting for 54% of BCRs. An IGKV3-15/IGKJ1-encoded stereotyped sequence homologous to WA RF accounted for 23% of BCRs. LPDs expressing KCDR3s homologous to anti-HCV E2 antibodies responded more frequently to the eradication of HCV by antiviral therapy (6/6 vs 1/6; p=0.015). These findings,

although limited by the small sample size, suggest that a stereotyped KCDR3 may predominantly shape anti-HCV specificity of BCRs, possibly providing a signature that may help identifying bona fide HCV-dependent LPDs (Genes and Immunity in press).

Beside investigating the molecular features of clonal B cells in LPDs, big effort has been made for the cellular and functional analysis of expanded B cell clones in LPDs to untangle the pathogenic mechanisms involved in disease non response or relapse after virus eradication. During the first year of the project we focused on the phenotypical and functional characteristics of rheumatoid factor (RF)-producing clonal B cells expanded in MC and NHL and followed their fate for a long follow-up after eradication of the virus, correlating our findings with MC vasculits response to DAA. Circulating B-cell clones, detected using flow cytometry either by the skewing of kappa/lambda ratio or by the expression of a VH1-69-encoded idiotype, commonly expressed in expanded clonal B cells in MC, were found in 18/45 patients, and in 17 of them persisted through the follow-up (18.5 months). There was no correlation between persistence of the B cell clones and response of the vasculitis or relapse and patients with complete clinical response and disappearance of cryoglobulins still presented circulating B cell clones. These observations suggest that "dormant" B cells persist long after viral eradication and that may be reactivated by events, as infections or onset of cancer that perturb B-cell homeostasis and can give rise to the relapse of cryoglobulinemic vasculitis (Visentini M. et al Liver International 2019).

To untangle this fascinating pathogenic hypothesis we performed several in vitro experiments analyzing the role of immune complexes that usually increase in vivo during infections and cancer, in the activation of RF+ B cells. We selected 6 MC patients, which had circulating monoclonal B cells and investigated the capacity of anti-Ig (F(ab′)2 anti-human Ig) or of ICs (heath-aggregated human IgG) to induce CpG-driven proliferation in patients' VH1-69pos clonal and VH1-69neg normal B cells. Stimulation with ICs alone did not induce proliferation but, similarly to anti-Ig, restored the capacity of VH1-69pos B cells to proliferate in response to CpG. We then analyzed the expression of TLR9 mRNA in clonal B cells to test if unresponsiveness to CpG alone was due to low TLR9 expression and surprisingly we found that VH1-69pos B cells constitutively expressed high TLR9 mRNA, suggesting that these cells could present the phenomenon of TLR tolerance (Poovassery JS et al. J Immunol 2009;183:2974-83). This consists of the fact that in immune cells stimulation of a TLR renders this, as well as other TLRs, unresponsive to further stimulation. In the case of B cells, it has been shown that prestimulation of TLR7 results in hyporesponsiveness of TLR7 and of TLR9 to restimulation, and that TLR responsiveness can be restored by BCR signaling through the phosphatidylinositol 3-kinase (PI3K)/AKT pathway. To support the hypothesis of a rescue from TLR-tolerance we investigated whether the PI3K/AKT pathway was intact in these cells. Stimulation with anti-Ig increased phosphorylated AKT (pAKT) levels in both VH1-69pos and VH1-69neg B cells, whereas stimulation with ICs induced pAKT only in VH1-69pos cells. Thus, the PI3K/AKT BCR signaling axis, which is involved in the rescue from TLR9 tolerance in B cells, is intact in clonal B cells of MC patients and can be triggered by the binding of ICs (manuscript subitted).

Finally we studied also essential mixed cryoglobulinemia (EMC) that is clinically and immunologically similar to HCV MC, but is idiopathic and apparently not related to infections. Molecular analysis of immunoglobulin genes rearrangements revealed circulating B-cell clones in about half of patients, on average of smaller size than those found in HCV-MC patients. Sequence analysis showed usage of the same stereotyped RF-encoding B-cell receptors frequently expressed in HCV-MC and in primary Sjogren's syndrome. B-cells with low expression of CD21 (CD21low) and unusual homing and inhibitory receptors were increased in EMC and in HCV-MC, but at a significantly lower extent in the former. The CD21low B- cells of EMC and HCV-MC patients shared functional features of exhaustion and anergy, namely reduced proliferation upon ligation of TLR9, high constitutive expression of phosphorylated ERK, and proneness to spontaneous apoptosis. These findings suggest a common pathogenic mechanism in EMC, HCV-MC consisting of autoantigen-driven clonal expansion and exhaustion of selected RF-producing B-cells (Del Padre M. Clin Exp Rheumatol. 2020).

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"UNDER 45" RESEARCH PROJETCS

2 YEARS PROJECTS LED BY UNDER 45 YEAR OLD RESEARCHERS – CALL 2019

FIRST SIX MONTHS REPORTS

IDENTIFICATION OF TRANSCRIPTIONAL PROGRAMS IN INNATE LYMPHOID CELLS UNDERLYING TUMOR PATHOGENESIS

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Innate lymphoid cells (ILCs) comprise a heterogeneous family of cells localized on the environmental interfaces to ensure protection against bacterial and viral infections. Similarly to T helper cells, several subsets of ILCs produce specific patterns of cytokines and, accordingly, are divided into five prototypical subsets. NK cells and ILC1 are characterized by the production of interferon (IFN)-γ; while ILC2 and ILC3 produce interleukin (IL)-5/13 and IL-17/22, respectively. Finally, lymphoid tissue-inducer (LTi) cells are also included within the ILC family and are characterized for their ability to determine the formation of secondary lymphoid structures (*Vivier E et al., Cell, 2018*). ILCs play key roles in regulation of the equilibrium of the gut mucosa, and are considered major amplifiers of inflammation. Loss of integrity of the gut epithelial barrier can lead to several pathologies, including inflammatory bowel disease (IBD) and, in the worst-case scenario, cancer (*Beaugerie L et al., N Engl J Med, 2015*).

Colorectal cancer (CRC) is the second most common cancer in women and third in men and, overall, the fourth cause of tumor death world-wide. The etiology of CRC is heterogeneous and depends on both environmental and genetic factors leading to a stepwise accumulation of DNA alterations in the gut epithelial cells. The mutational landscape of CRC comprises mutations in the APC gene (70-80% of CRC patients), KRAS (30-50%), TP53 (40-60%), and in genes associated with the TGF-ß and PI3K signaling pathways. The majority of patients develop sporadic CRC, while 10-30% of the cases present family history, and less than 5% have hereditary forms of CRC. As mentioned above, the chronic inflammation in patients with IBD represents a significant independent risk factor for CRC development (*Torre LA, et al. CA Cancer J Clin, 2015*). Due to the complexity of CRC pathogenesis, the identification of novel cellular and molecular pathways represents a pressing unmet need.

Objectives and Results

In this project, we propose that a deeper understanding of the tumor-infiltrating ILC subsets and the signals involved in shaping their effector functions will allow us to unveil novel mechanisms underlying CRC pathogenesis and progression. The research program aims to address this aspect by achieving the following two objectives:

1. to identify protective/pathogenic phenotypes in tumor-infiltrating ILCs;
2. to discriminate the factors involved in regulation of ILC plasticity/functions associated with tumor progression.

We are employing a broad approach, which combines cutting-edge flow-cytometry and next-generation sequencing technologies with models of CRC in established and novel mouse strains and CRC patient samples. Different mouse models of CRC have been described recapitulating several traits of the human disease. During the first 6 months of the "Under 45" research project, we employed three different conventional mouse models of CRC, comprising: *1)* a model for colitis-induced CRC, initiated by an intraperitoneal (*ip*) injection of a single dose of azoxymethane (AOM) and followed by administration of dextran sulfate sodium (DSS) in the drinking water for one week (4 cycles); *2)* a model for sporadic CRC, consisting on AOM administration (6 cycles, once a week); and, *3)* a genetic model for CRC by using *Apc(Min/+)* mice, which spontaneously develop polyps. In these experiments, we have analyzed the phenotypes of ILCs by using 18-parameter flow cytometry. In particular, we assessed cells isolated from tumors, as well as from the colonic mucosa of treated mice, after tumor excision and mice left untreated. Our preliminary results shed light on the drastic alterations of the ILC phenotypes occurring during the onset of inflammation and in the settings of cancer. Next, we aim to deconvolute the complexity of ILC transcriptional states in the context of CRC pathogenesis by using Next Generation Sequencing (NGS) approaches, enabling us to discriminate protective/pathogenic pathways regulating ILC functions in inflammation and cancer settings.

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SENSING ARGININE THROUGH THE VENUS FLY TRAP DOMAIN TO CONTROL c-di-GMP LEVELS IN PSEUDOMONAS AERUGINOSA: MOLECULAR MECHANISM AND METABOLIC EFFECTS OF SIGNAL TRANSDUCTION.

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This project aims at unveiling how the opportunistic human pathogen *Pseudomonas aeruginosa* controls the second messenger c-di-GMP (3',5'-cyclic diguanylic acid) in response to the nutrient Arginine. The intracellular levels of c-di-GMP are related to biofilm formation, a multicellular lifestyle difficult to be eradicated. Among nutrients, Arginine represents one key metabolite in biofilm formation being both at the crossroad of many metabolic processes and associated to chronic infections, biofilm/virulence and antibiotic resistance.
We recently found that *P. aeruginosa* is able to perceive environmental Arginine to decrease the intracellular levels of c-di-GMP via the RmcA (Redox regulator of c-di-GMP) protein (Paiardini *et al.*, 2018).
The final goal of this proposal is to gain details on the mechanism of Arginine-mediated signal transduction controlling c-di-GMP hydrolysis both in terms of molecular activation and of the metabolic re-programming cascade to finally identify the proper environmental conditions to augment biofilm dispersal.

RmcA is a multidomain membrane protein (Figure 1) harbouring a Venus Fly Trap (VFT) domain devoted to periplasmic Arginine binding and a cytoplasmic portion containing PAS-LOV domains linked to the diguanylate cyclases (GGDEF) and the phsphodiesterases (EAL) catalytic tandem, where the hydrolysis of c-di-GMP occurs (Mantoni *et al.*, 2018).

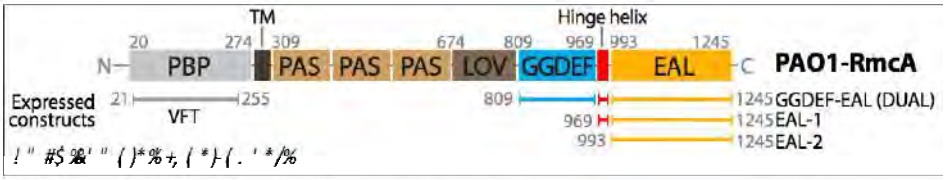

Figure 1. RmcA domain organization

Given the complexity of the protein and the possible metabolic pathways related to RmcA activation, we divided our study into two main tasks focused on the molecular and metabolic characterization of the RmcA activity, respectively.

TASK 1: Mechanism of VFT-mediated Arginine sensing and signal transduction in RmcA.

The biochemical characterization of this complex protein has been tackled by looking to the structure-function relationship of isolated domains.

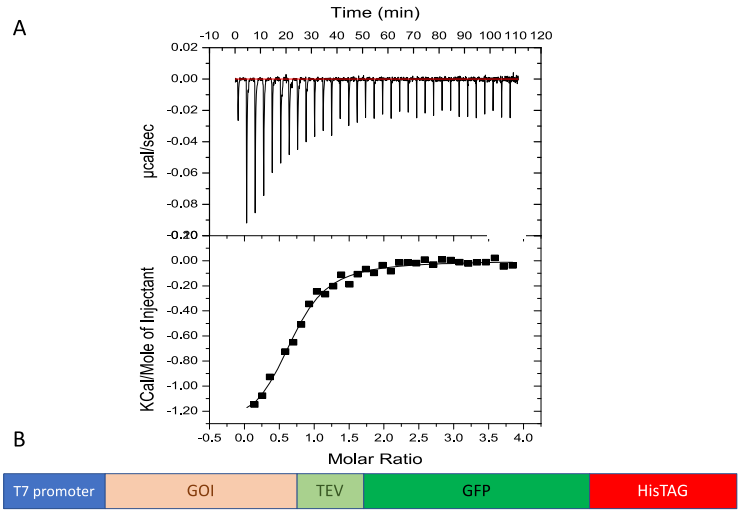

Figure 2. (A) Binding of L-ornithine to purified VFT. Binding of L-ornithine to VFT was assayed by Isothermal Titration Calorimetry (ITC), by titrating a VFT solution with different amounts of L-ornithine. The upper panel shows the heat exchange in time; in the lower panel the normalized enthalpy exchange (black squares, reported as Kcal/Mol of injectant) The single binding site equation yields astoichiometry of binding of 0.8 and a Kd=7.9 µM. (B) Architecture of the synthetic gene construct. GOI: gene of interest; TEV: signal sequence for proteolytic cleavage by TEV protease; GFP: green fluorescent protein.

During these first six months of the project we investigated the capability of the VFT to recognize metabolite(s) other than Arginine. We found that the VFT displays a comparable affinity for the ornithine amino acid (Figure 2A), indicating that the RmcA activity could be related to nitrogen assimilation or anaerobic respiration of these amino acid. By integrating this evidence and Bioinformatics predictions, it is likely that this domain is able to recognize also citrulline, being the aminogroup of the side chain crucial to promote stacking interactions with the binding pocket residues. On the other hand, the carboxy terminal group of the amino acids is crucial for stabilising the binding to the VFT domain *via* polar interactions; in light of this, unlikely polyamines could be genuine ligands of such receptor, being the carboxy group missing. Binding experiments are currently ongoing to validate this model.

To better characterize the role of VFT in transducing the signal upon signal binding, we proposed in the project to characterize a prototype protein harboring the VFT-GGDEF-EAL domains. In this first period, we designed an *ad hoc* construct to facilitate the detection and the purification of the protein (Figure 2B). We envisaged to remove the GFP tag (for detection during purification) with unique restriction sites, once the purification protocol will be setted). The construct, as designed, will be useful also for cloning other membrane protein. The gene, as synthetic construct, has been delivered very recently and gene sequence validation is currently ongoing. We specifically avoided to order an *E. coli* optimized sequence, given the possible interference of this strategy with correct folding (Parret *et al.*, 2016).

The biochemical characterization has been carried out also on the cytoplasmic part of the protein. In particular, the role of FAD in controlling the LOV domain over the catalytic GGDEF-EAL moiety has been investigated in detail.

We produce two different constructs, named short and long, both including the LOV domain and the latter harboring an extra N-terminal helix. Interestingly, the addition of only 15 residues in the long construct leads to a monodisperse protein, contrary to the short counterpart, where aggregates, tetramer and dimer were populated (Figure 3).
Preliminary data on the long construct shows an effect of FAD and GTP on catalysis which is significant but different to that observed with the short protein. This heterogeneity in the allosteric behavior of the protein strongly confirm the extreme plasticity of the GGDEF-EAL protein, which raises some concern on the design of truncated protein. In light of this, we have characterized the reactivity with GTP of 4 different GGDEF truncated proteins and identified the possible weakness and caveats in designing truncated version of a GGDEF-containing protein (Manuscript in preparation).

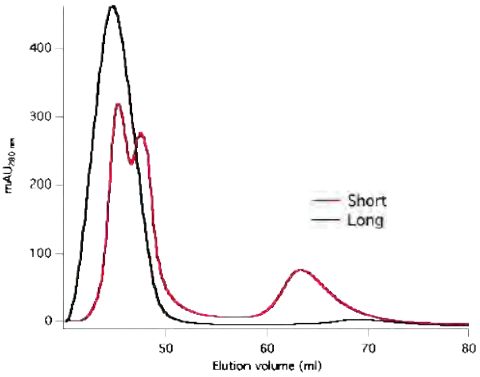

Figure 3. SEC elution profile (Superdex S-200) of the short and the long LOV-GGDEF-EAL constructs.

The structural determinants of the FAD/LOV interaction has been investigated by means of protein engineering experiments. A conserved glutamine has been substituted with alanine and the corresponding protein loses the capability to bind and respond to FAD (Figure 4).

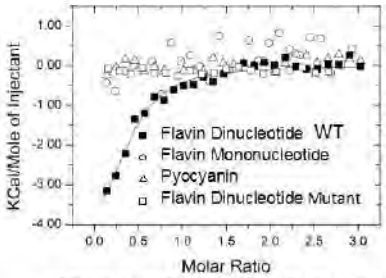

Figure 4. Binding of FAD to the LOV wt and mutant domain, assayed by Isothermal Titration Calorimetry (ITC). As negative control binding of FMN and Pyocyanin to LOV were also included

Given that bioinformatic analysis suggested that the protein may adopt different conformations depending on the redox state of the FAD cofactor we have also identified the proper conditions to carry out the experiments with $FADH_2$: a special treatment to remove oxygen has been done in the presence of little amount of reductant. We were able to promote FAD reduction without interference of the reductant with the c-di-GMP CD spectroscopic signal. This preliminary set up is useful to carry out experiments under anaerobiosis, a condition required to populate $FADH_2$ (experiments are currently ongoing).

In order to understand the mechanism of c-di-GMP consumption in response to Arginine, we are carrying out preliminary set up to perform kinetics on the full-length protein. This part of the project is taking advantage of a key collaboration with Prof. Lars Dietrich from the Columbia University (US): he is a long-lasting expert of anaerobiosis and redox metabolism of *P. aeruginosa* (PA14) and he has carried out interesting characterization on RmcA in terms of its role in controlling *P. aeruginosa* morphology and phenazine sensing (Okegbe C *et al.*, 2017). We have obtained the proper set up to

detect c-di-GMP in the presence of those detergents required to handle the membrane protein and we are ready to start this part of the study.

TASK 2: Role of RmcA in controlling the metabolic re-programming in P. aeruginosa under different environmental conditions.
A first characterization of the role of RmcA in *P. aeruginosa* was carried out on PAO1 strain. We found that the RmcA mutant, while it can grow using L-Arginine as the sole source of carbon (although with altered c-di-GMP levels), it has trouble growing when L-Arginine serves also nitrogen supply. In line with the idea that RmcA is involved in nitrogen assimilation homeostasis, we have preliminary evidences suggesting that the glutamate dehydrogenase activity is sustained in the mutant mainly by the Arginine-independent isoform, as compared to the wildtype.
Given that recent data suggest that PAO1 could be not ideal to be used for studying the anaerobic/redox metabolism of *P. aeruginosa* (Crespo *et al.*, 2017), we decided to move to PA14, whose RmcA superposes with that of PAO1 but it is ideal for redox characterization. For this, the collaboration with Prof. Lars Dietrich will be strategic. Future set up will be investigated to understand the role of the RmcA-mediated Arginine sensing and metabolic re-programming.
The most relevant results will be discussed during the next 25th European Nitrogen cycle meeting which will be held in Sapienza in September; the PI of this project is directly involved in the organization in collaboration with other colleagues coming from European Universities.

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THE ROLE OF THE HIPPO PATHWAY IN THE DEVELOPMENT OF ENDOTHELIAL DYSFUNCTION AND VASCULAR DAMAGE IN RESPONSE TO METABOLIC ABNORMALITIES

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Introduction

Cardiovascular diseases still represent the major causes of death in the Western Countries (1). Endothelial dysfunction is the initial vascular pathological event leading to the subsequent development of atherosclerosis and overt cardiovascular diseases such as myocardial infarction, stroke and peripheral artery disease (2). Endothelial cells regulate the tone of blood vessels producing various vasoactive molecules, including nitric oxide (NO), prostacyclin, endothelin, superoxide anions and angiotensin II. These endothelial-derived molecules can also regulate platelet aggregation, vascular inflammation and oxidative stress, contributing to the progression of atherosclerotic disease (2).

Main cardiovascular risk factors directly promote an impairment of endothelial function. Among risk factors, diabetes mellitus (DM) is a highly morbid condition strictly associated with the development of cardiovascular diseases (3). Endothelial dysfunction plays a major role in the development of macroangiopathy and atherosclerosis in diabetic patients leading to the early onset of adverse cardiovascular events (3). In response to the metabolic alterations associated with diabetes, such as high glucose and lipid levels, vascular endothelial cells lose the major vasodilating properties with the consequent development of endothelial dysfunction (3). In particular, there is an increase in oxidative stress and a reduction in the bioavailability of NO, which elicits important vasodilating, antioxidant and anti-inflammatory actions. It is therefore necessary to find new pharmacological therapies that can prevent and treat diabetes-induced endothelial damage in order to reduce the onset of atherosclerosis and the consequent development of cardiovascular diseases.

In recent years a new pathway has emerged as a possible molecular mediator involved in the development of cardiovascular diseases: The Hippo pathway. The Hippo pathway is an evolutionarily conserved intracellular signaling pathway involved in the regulation of cell survival and growth (4). It was firstly discovered in Drosophila and most of the components of the pathway have a homologue in mammals. A key component of the Hippo pathway is the mammalian sterile 20-like kinase 1 (MST1), a serine threonine

kinase belonging to the STE20 family and homologue of the Drosophila Hippo. MST1 is activated by various stimuli, including oxidative stress, and when activated, MST1 phosphorylates and activates the Large Tumor Suppressor Kinases 1/2 (Lats1/2). Lats1/2 phosphorylate in turn the co-transcription factor Yes- associated protein 1 (YAP1), a promoter of cell survival and growth, which is consequently exported out of the nucleus and degraded (4).
Previous work showed that Hippo signaling induces apoptosis and inhibits cell growth. Based on these functions, the pathway plays a critical role in controlling organ homeostasis in mammals in unstressed conditions by maintaining the equilibrium between cell death and proliferation. However, Hippo signaling activation also contributes to cell death and tissue injury in response to stress (4). Accumulating lines of evidence also demonstrated that the Hippo pathway plays significant functions in the heart, particularly during stress. MST1 overexpression in cardiomyocytes in vivo leads to the development of cardiomyocyte apoptosis, dilated cardiomyopathy and heart failure (5). In addition, it was previously demonstrated that endogenous MST1 is activated in response to myocardial ischemia/reperfusion, chronic ischemic remodeling and pressure overload, thereby contributing to the development of cardiac injury (5-8). Hippo signaling genetic inhibition was also shown to promote heart regeneration after injury through the activation of YAP1 (9). Finally, previous work demonstrated that MST1 activation contributes to the development of diabetic cardiomyopathy (10). Overall, this evidence suggests that inhibition of MST1 may be a potential therapeutic intervention for the treatment of cardiovascular diseases. However, although Hippo pathway activation in macrophages was found to be implicated in atherosclerosis formation, the specific role of the Hippo pathway in endothelial cells and in the development of endothelial dysfunction and vascular damage in response to metabolic derangements, such as diabetes, still needs to be fully defined.

Results

MST1 overexpression reduces endothelial cell viability and angiogenesis

Human umbilical vein endothelial cells (HUVEC) were infected with an adenovirus overexpressing MST1 (Ad-MST1) for 48 hours. An adenovirus overexpressing ß-galactosidase (Ad-LACZ) was used as a control. HUVEC with MST1 overexpression showed a 4-fold increase in apoptosis with respect to control cells ($p<0.001$). Similarly, MST1 overexpression also caused a reduction of cell viability and angiogenesis (Figure 1A-B).

MST1 inhibition promotes capillary-like structure formation in HUVEC after metabolic stress

We evaluated the effects of cellular stress on endogenous MST1 activation. We found that HUVEC exposure to high glucose or oxidized LDL levels significantly increased MST1 autophosphorylation, a marker of its activation (Figure 1C). Furthermore, to

understand whether MST1 activation contributes to endothelial damage during stress, we overexpressed a dominant negative form of MST1 (kinase dead) in HUVEC and then we subjected the cells to hyperglycemia. We found that HUVEC capacity to form capillary-like structure was rescued by MST1 inhibition under this condition (Figure 1D). Finally, we found that MST1 inhibition partially rescued the deleterious effects of these stress conditions on acetylcholine-induced vasorelaxation suggesting that MST1 inhibition may reduce stress-induced endothelial dysfunction (Figure 1E).

MST1 overexpression promotes ROS production and activates RAC1- NOX2 axis

We investigated whether MST1 regulates ROS production and NOX2 activity. H2O2 production and NOX2 activation status were evaluated in HUVEC infected with Ad-MST1 and Ad-LACZ. MST1 overexpression increased H2O2 production and NOX2 activity (Figure 2A-B). Interestingly, MST1 overexpression significantly increased the activation levels of RAC1, as
indicated by the increased GTP- loading (Figure 2C).

In addition, MST1 overexpression reduced the interaction of RAC1 with LyGDP, an endogenous RAC1 inhibitor (Figure 2D). Overall, these data indicate that MST1 activation promotes ROS production and RAC1-NOX2 activation. Finally, we found that MST1 inhibition by DN-MST1 overexpression reduces oxidative stress in mesenteric arteries incubated with oxidized LDL *ex vivo* (Figure 2E).

Figure

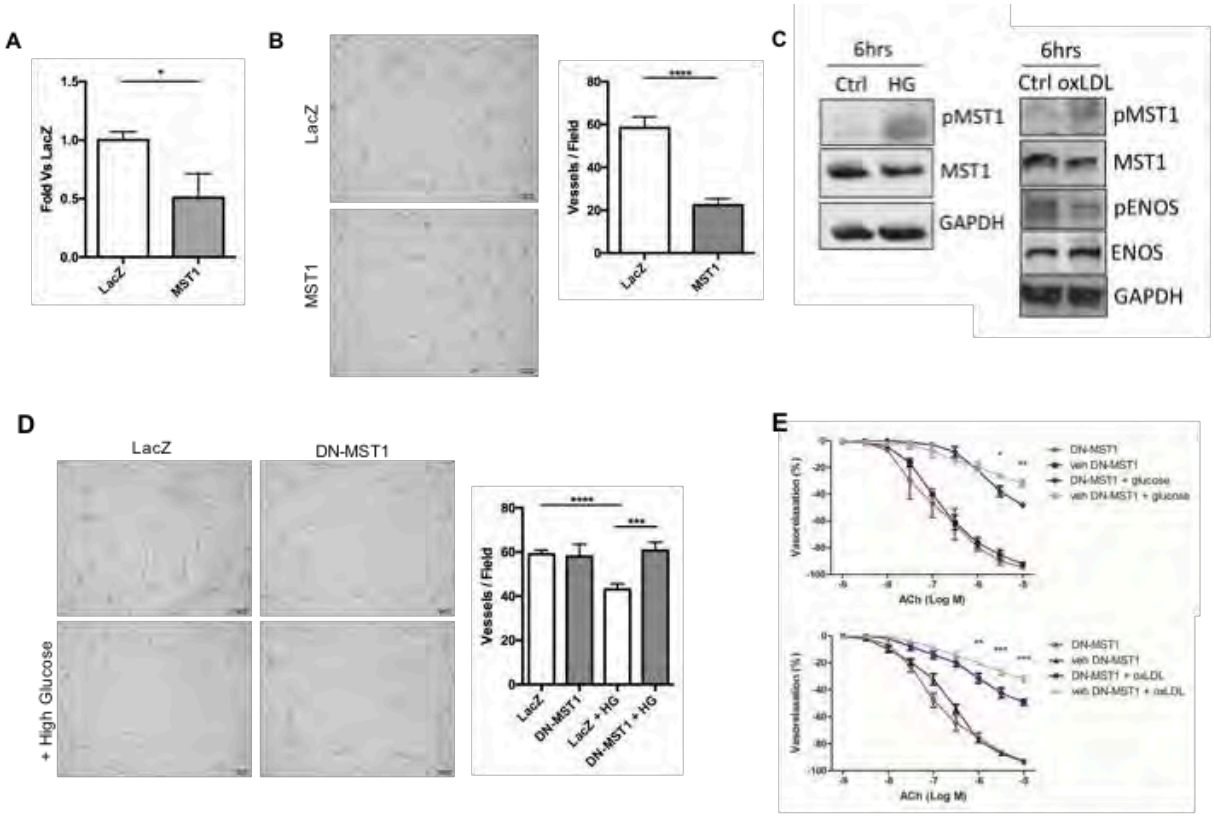

Figure 1. (A) HUVEC cell viability after 48 hours of Ad-MST1 and Ad-LacZ infection by MTS assay (*p=0.02). (B) Matrigel assay in HUVEC previously infected for 48 hours with Ad-MST1 and Ad-LacZ (N=6 ****p<0.0001). (C) Western Blotting analysis of ENOS, phospho-ENOS, MST1, phospho-MST1 in HUVECs treated with 25mM of glucose or 30 µg/mL of oxidized LDL for 6 hours. (D) Representative optical images of HUVECs infected with Ad-DN-MST1 and Ad-LacZ for 48 hours, treated with glucose (HG, 25mM) for 24 hours and then plated on Matrigel in EBM medium (****p<0.0001 LacZ vs LacZ + HG,***p=0.004 LacZ + HG vs DN-MST1 + HG). (E) Endothelial-dependent vasorelaxation of mesenteric arteries from C57BL/6J mice with and without (Vehicle) DN-MST1 expression, before and after 3 hours of glucose (25mM) treatment or 90 minutes of oxidized LDL treatment (30 µg/mL). N=5.

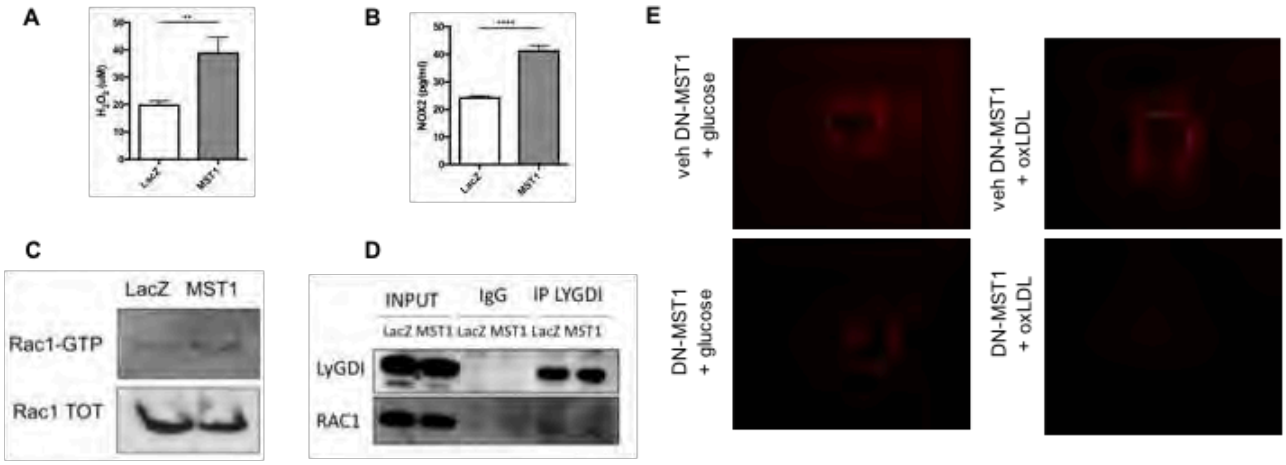

Figure 2. (A) H_2O_2 levels by a colorimetric assay in HUVECs infected with Ad-MST1 and Ad-LacZ for 48 hours (N=8 **p=0.006). (B) NOX2 activation was assessed by an ELISA assay in HUVECs infected with Ad-MST1 and Ad-LacZ for 48 hours (N=8 ****p<0.0001). (C) Rac1-GTP levels (the active form) were assessed in HUVECs infected with Ad-MST1 and Ad-LacZ for 48 hours. (D) Immunoprecipitation (IP) assay of HUVECs infected with Ad-MST1 and Ad-LacZ for 48 hours. Cell lysates were immunoprecipitated with anti-LyGDI antibody and analysed by immunoblotting with the indicated antibodies. (E) Superoxide anion levels by DHE staining in sections of mouse mesenteric arteries with or without (Vehicle) DN-MST1 expression and treated with glucose (25mM) or oxidized LDL (30 µg/mL).

Conclusions

These results indicate that MST1 overexpression reduces endothelial cell viability and function in vitro. On the other hand, its inhibition reverses the deleterious endothelial effects of hyperglycemia and oxidized LDL. Interestingly, we found that MST1 overexpression promotes ROS production and RAC1-NOX2 activation. This result is particularly relevant since NADPH oxidase-derived ROS were previously shown to induce endothelial dysfunction in response to stress (11). Among different NOX isoforms, previous work demonstrated that NOX2 inhibition reverses diabetes- induced endothelial dysfunction. Inhibition of RAC1, a small GTP-binding protein that significantly activates NOX2, was also shown to reduce endothelial dysfunction in response to diabetes and in human patients (12- 13)

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THE SWEET LINK BETWEEN BRAIN DYS-METABOLISM AND COGNITIVE DECLINE: A NOVEL ROLE FOR PROTEIN O-GlcNAcylation.

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In recent decades, the increase in both life expectancy and incidence of metabolic diseases has caused the augmented occurrence of a condition named dys-metabolic ageing. The connection between ageing and altered metabolism in the development of cognitive decline has attracted the attention of researchers in these fields. From an epidemiological and pathophysiological point of view, Alzheimer Disease (AD) and type-2 diabetes (T2D) appear to be intimately connected as indicated by the higher incidence of cognitive impairment in diabetic patients and by the inverse relationship between elevated blood glucose levels and hippocampal volume. It is now established that nutrients impact on the structural and functional integrity of hippocampus expressing receptors for nutrient-related signals. Emerging evidence suggests that metabolic dysregulation affects cognitive function and increases the incidence of cognitive decline (CD). Experimental models of overnutrition and metabolic diseases (e.g., obesity and insulin resistance) show severe learning and memory defects. High fat diet (HFD) is the most commonly used experimental model of obesity and insulin resistance, almost completely resembling the hallmarks of metabolic syndrome identified in humans. It also causes detrimental effects on brain function, but the molecular mechanisms underlying the impact of nutrient excess on cognitive function are still poorly understood. Recent studies show that HFD suppresses the expression of glucose transporters (GLUTs) at the blood-brain, as well as in neurons, resulting in the reduction of glucose uptake, which represent an early step in neuronal damage. In this regard, AD was recently proposed to be a form of CD caused by metabolic dys-homeostasis. Intriguingly, during AD glucose hypometabolism precedes the appearance of clinical symptoms leading to the downregulation of O-GlcNAcylation, a reversible protein post-translational modification (PTM), which consist in the attachment of an N-acetylglucosamine moiety to serine or threonine residues. Interestingly, the mutual relationship with phosphorylation gives to this modification the ability to modulate various signalling pathways involved in neuron development and homeostasis. Protein O-GlcNAcylation depends on the availability of glucose and therefore constitutes a powerful mechanism by which cellular activities are regulated according to the nutritional environment of the cell. Indeed, it is modulated by

a transferase, OGT, and a hydrolase, OGA, whose activation require energy supply and substrate production through the hexosamine biosynthesis pathway (HBP). Aberrant O-GlcNAcylation is known to be implicated in several human pathologies including type-2 diabetes and Alzheimer disease. Recent studies from our laboratory observed the reduction of O-GlcNAcylation in the hippocampus of an AD mouse model, supporting a role in controlling brain glucose utilization, mitochondrial function and the development of CD.

The idea behind this project is that the homeostasis of glucose metabolism in the brain is necessary for the maintenance of learning and memory. Our experimental hypothesis is that diet-driven metabolic changes in the brain impinge on the HBP interfering with protein O-GlcNAcylation, which may lead to accelerated brain ageing.
To carry-out the experimental part of the project we fed C57 mice for 6 weeks (short-term) with HFD (60% of caloric intake from fat) and compared them with C57 fed with standard diet (SD). At first, we analyzed the impact of HFD on total protein O-GlcNAcylation and on total protein phosphorylation on Ser and Thr residues. As expected, our results show an inverse trend between these two PTMs. Indeed, a significant reduction of total GlcNAc levels was coupled with the increase of phosphorylated residues in HFD mice. This initial finding supports the notion that HFD alters brain and functionality affecting protein O-GlcNAcylation/phosphorylation ratio. Subsequently we analyzed the levels and activity of the two enzymes regulating the O-GlcNAcylation cycling. The analysis of OGA demonstrated no differences concerning expression levels and activity between HFD and SD mice, while the analysis of OGT demonstrated a decrease of its expression levels in HFD mice. This result is in line with previous studies that reported OGT alterations in dys-metabolic condition in the brain. In order to understand the effects of aberrant protein O-GlcNAcylation/phosphorylation ratio in the development of the pathological markers of AD-like dementia we analyze APP and tau PTMs. APP is the precursor protein of amyloid β (Aβ) peptide and its abnormal cleavage, by γ-secretase, may depend on phosphorylation levels. We show, in HFD mice, the reduction of O-GlcNAcylated APP, which promoted the increase of phosphorylation and conceivably the formation of Aβ. Furthermore, HFD mice show the reduced O-GlcNAcylation of tau protein, which makes it prone to hyperphosphorylation allowing the development of neurofibrillary tangles. Currently, we are analyzing the functionality of the HBP and the levels and activity of GFAT1, the rate-limiting enzyme of the pathway, to understand the molecular mechanisms that lead to reduced O-GlcNAcylation in HFD mice. In parallel, we isolated primary astrocyte from C57 mice and subjected these to insulin + palmitic acid (IPA) treatment for 24h. The analysis of total protein O-GlcNAcylation reported a reduction after IPA, as observed in HFD mice. We are currently characterizing astrocytes in terms of reduced glucose metabolism and altered HBP and subsequently we will test the ability of Thiamet G, an OGA inhibitor, to rescue protein O-GlcNAcylation and protect from nutrient-driven damage.

Our data demonstrated, so far, that HFD lead to unbalanced O-GlcNAcylation/phosphorylation ratio of total proteins and of tau and APP suggesting a role in tau hyperphosphorylation and in the increased production of Abeta. A reduction

of OGT but not of OGA protein levels is reported in the hippocampus supporting a role for this enzyme as a sensor for dys-metabolic conditions.

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AUTOCRINE TUMOR NECROSIS FACTOR SIGNALLING IN REGULATORY T CELL EXPANSION IN CANCER

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Tumor necrosis factor (TNF) is a pleiotropic cytokine traditionally considered a major actor in inflammation, however several studies have highlighted the capacity of TNF to exert also anti-inflammatory and immunomodulatory effects. It has been described that TNF promotes survival, proliferation and effector function of regulatory T cells (Treg), a CD4 T cell subset with immune suppressive function that constitutively express TNF receptor 2 (TNFR2), and can further upregulate it following activation. This occurs especially at the tumor site, where Tregs are expanded and activated. Notably, some pieces of evidence indicate that Tregs themselves are able to produce TNF and this has been hypothesized to support their proliferation through an autocrine loop.
The aim of this project is to dissect the possible role of TNF production by Treg on their own expansion and function, especially in the tumor context, where Tregs are potentially more responsive to this cytokine, and where TNF have been reported to play anti-tumoral as well as pro-tumoral functions.
First, we tested whether Treg-derived TNF played a role in Treg proliferation in vitro: in this setting, in the absence of any exogenous TNF source, an anti-TNF neutralizing Ab was able to suppress the proliferation of Tregs at a higher extent than conventional T cells (Tconvs), demonstrating that the Treg culture contained TNF that was required for proliferation.
Then, we analyzed the expression of TNF and TNFR2 in Tregs and Tconvs infiltrating murine tumors, induced by transplantation of a hepatocellular carcinoma or a colorectal adenocarcinoma cell line: TNFR2 and TNF were both upregulated by Tregs at the tumor site compared to the spleen; interestingly, these two molecules showed a mutually exclusive expression, suggesting the existence of two alternative states or subsets that either sensed or produced TNF.
To better characterize the features of these populations, TNFR2+ and TNFR2- Tregs were sorted by flow cytometry from the spleens of Foxp3YFPcre mice, carrying a reporter fluorescent protein in Tregs. After a brief restimulation ex vivo, TNFR2+ Tregs produced TNF at a significantly lower level than TNFR2- Tregs and Tconvs. When polyclonally stimulated in vitro for 4 days, both TNFR2- and TNFR2+ were able to upregulate

TNFR2. In particular, the increase of TNFR2 expression on TNFR2- Tregs was dependent on autocrine TNF, since it was blocked by the addition of a neutralizing anti-TNF antibody, and it could also be inhibited by the co-culture with TNFR2+ Tregs that probably sequestered the available cytokine. According to these data, TNFR2 expression can be modified in vitro and thus appears as a dynamic feature; conversely, we found that the two subpopulations maintained their different ability to produce TNF in vitro, suggesting certain stability in this capacity.

Through a flow cytometry-based analysis that allowed the concomitant assessment of TNF protein and TNF mRNA content, we observed that the two Treg subsets had the same TNF mRNA and differed only for the TNF protein levels: this finding prompted us to investigate the role of post-transcriptional events in inhibiting TNF translation in TNFR2+ Tregs. We focused our attention on miR146a, which plays important roles in Treg functions and has also been shown to regulate TNF mRNA in other cells: through flow cytometry, we could demonstrate that TNFR2+ Tregs contained significantly higher levels of this miRNA compared to TNFR2- Tregs and to Tconvs. Based on these results, we plan to investigate whether the blockade of miR146a, by means of an antagomir, can rescue TNF production in TNFR2+ Tregs.

Overall these data indicate that Tregs may exist in two functional states with different ability to produce or sense TNF, and that internal regulatory mechanisms may dictate the balance between the two **(Figure 1)**. Our next goal will be to study the proliferative capacity, the suppressive function, and the pro- or anti-tumor activities, of these two subpopulations when adoptively transferred into tumor-bearing mice.

In summary, our data demonstrate that Tregs may utilize the TNF-TNFR2 signaling pathway in the regulation of their own expansion, though autocrine as well as paracrine events. Understanding the mechanisms governing this pathway may help the development of novel Treg manipulation approaches for cancer therapy.

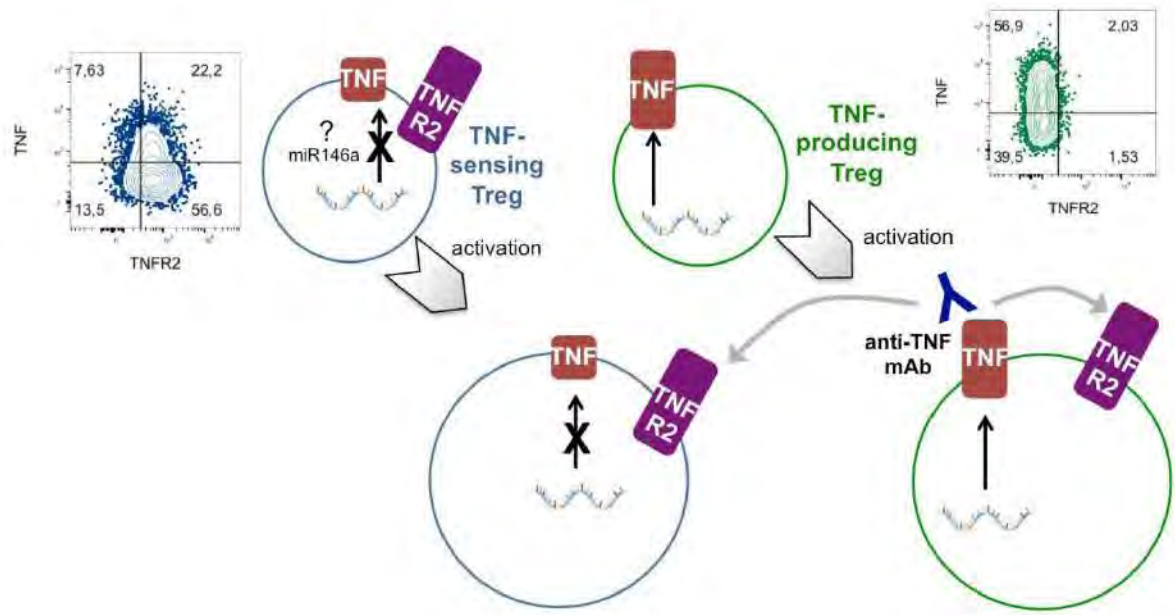

Figure 1. Our working hypothesis about two functional states of Tregs.
Tregs contain two subsets, one equipped to sense TNF (being TNFR2+ and producing low levels of TNF) and the other one equipped to produce TNF (being TNFR2- and producing high levels of TNF). Post-transcriptional regulation by miR146a could be responsible for the differential TNF production by the two subsets. When cultured in vitro, TNFR2- Tregs upregulate TNFR2 through an autocrine TNF loop, which is inhibited when TNF availability was restrained by the addition of a neutralizing antibody or by the coculture with TNFR2+ Tregs. Conversely, TNFR2+ Tregs maintained in vitro their low TNF production.

Publications

Pacella I, Piconese S. ***Immunometabolic Checkpoints of Treg Dynamics: Adaptation to Microenvironmental Opportunities and Challenges.*** *Frontiers Immunol.* 2019 10:1889. IF: 5.511

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STUDY OF THE ROLE OF RNA-BINDING PROTEINS IN THE NEURODEGENERATIVE DISEASE AMYOTROPHIC LATERAL SCLEROSIS

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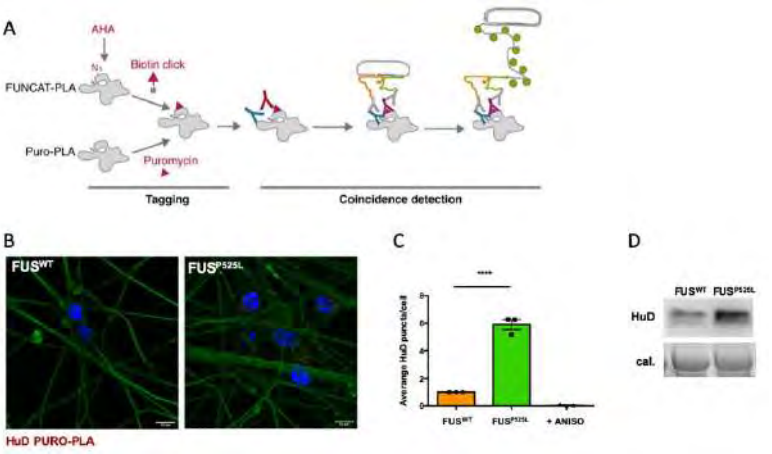
The RNA-binding protein FUS is mutated in a subset of patients affected by the motor neuron disease Amyotrophic Lateral Sclerosis (ALS). The general goal of our project is to gain insights into the toxic functions acquired by mutant FUS, with a specific focus on the aberrant crosstalk between mutant FUS and other RNA-binding proteins (RBPs). We take advantage of human induced Pluripotent Stem Cells (iPSCs) carrying a pathogenic mutation in the FUS gene, which can be differentiated into motor neurons.
This annual report is relative to the first six months of the project. During this period, we have been focused on the characterization of the molecular mechanisms underlying the dysregulation of a neural RNA-binding protein, HuD (also known as ELAVL4), in mutant FUS motor neurons (Aim 1). We have also analysed the proteome of motor neurons derived from FUS wild-type or mutant iPSCs (Aim 2).

The Aim 1 of our proposal is to define the molecular mechanisms and functional consequences of mutant FUS and HuD interplay. In a previous work (De Santis et al., Cell Reports 2019; doi:10.1016/j.celrep.2019.05.085), we had shown mutant FUS binding on HuD 3'UTR. In the first six months of this project, we have gained important insights on the consequences of this interaction. Thanks to an ongoing collaboration with Dr. Pietro Fratta (UCL, London, UK) we have performed Puro-PLA, a technique that couples puromycylation with the proximity-ligation assay to visualize newly synthesized proteins and monitor their origin in situ (tom Dieck et al., Nat Methods 2015; doi:10.1038/nmeth.3319; Fig. 1A). Puro-PLA was performed on FUS mutant and wild-type iPSC-derived motor neurons using and anti-HuD antibody (Fig. 1B). We found increased number of HuD sites of translation in mutant FUS cells (Fig. 1C), leading to aberrant increase of HuD protein levels (Fig. 1D). These findings have been confirmed in primary MNs from a FUS-ALS mouse model developed in the Fratta lab.

Figure 1. (A) Schematic representation of the Puro-PLA method (tom Dieck et al., Nat Methods 2015). (B,C)

Representative image and quantification of HuD Puro-PLA in iPSC-derived motor neurons with a FUS WT or mutant (P525L) genetic background. (D) Western blot analysis of HuD protein levels in the same cells.



We are currently addressing a possible mechanism underlying this alteration. We hypothesize that a competition for HuD 3'UTR binding between mutant FUS and inhibitory RBPs exists in motor neurons. Thus, mutant FUS might hamper the activity of one or more RBPs that have the function of keeping low levels of HuD, in normal conditions, through 3'UTR binding and translation inhibition (at the level of initiation or elongation). An important functional consequence of increased HuD protein levels is upregulation of its target transcripts. For instance, it was previously shown that HuD, by 3'UTR binding, stabilizes the mRNA encoding for the neurotrophic factor NRN1 (Neuritin1, also known as CPG15) (Akten et al., PNAS 2011; doi: 10.1073/pnas.1104928108). We indeed found increased levels of NRN1 in FUS mutant motor neurons and in FUS wild-type motor neurons overexpressing exogenous HuD (Fig. 2A-C). Interestingly, by taking advantage of microfluidics devices, we found increased axon branching and arborization in FUS mutant motor neurons (Fig 2D-E). Also in this case, such findings have been confirmed in the mouse model. This phenotype might be a consequence of increased NRN1. We are currently addressing whether a causal link exists between FUS mutation, increased HuD protein, increased NRN1 and aberrant axon branching.

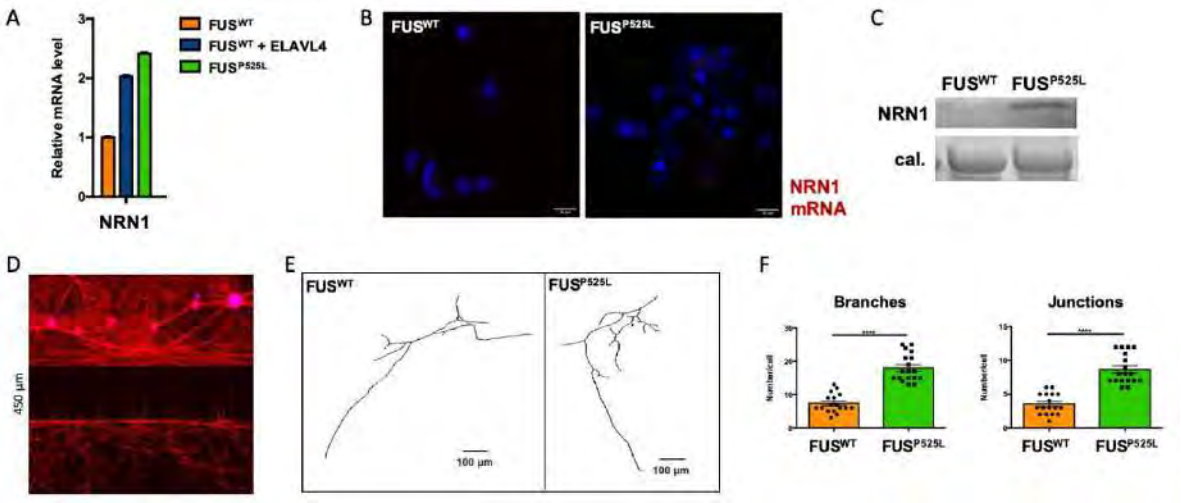


Figure 2. (A) Real-time qPCR analysis of NRN1 mRNA levels in iPSC-derived motor neurons with a FUS WT or mutant (P525L) background and in FUS WT cells overexpressing HuD (ELAVL4). (B) RNA-FISH with a NRN1 probe. (C) Western blot analysis of NRN1 protein levels. (D) iPSC-derived motor neurons plated into one chamber of microfluidics devices with 450 µm microgroove barrier (Xona Microfluidics) and stained with an anti-TUJ1 antibody (red). (E-F) Analysis of the number of branches and junctions in terminal axons of iPSC-derived motor neurons.

The Aim 2 of our proposal is to globally assess the impact of mutant FUS on its interactors. We have established a collaboration with Dr. Andrea Armirotti (Analytical Chemistry Facility; Fondazione Istituto Italiano di Tecnologia, IIT) who has extensive expertise in label-free proteomics analysis by mass-spectrometry. High-resolution LC-MS/MS analysis of the proteome of pure populations of wild-type and FUS mutant human motor neurons, derived from isogenic iPSCs, revealed altered expression of proteins involved in the cytoskeleton, among other categories (Fig. 3).

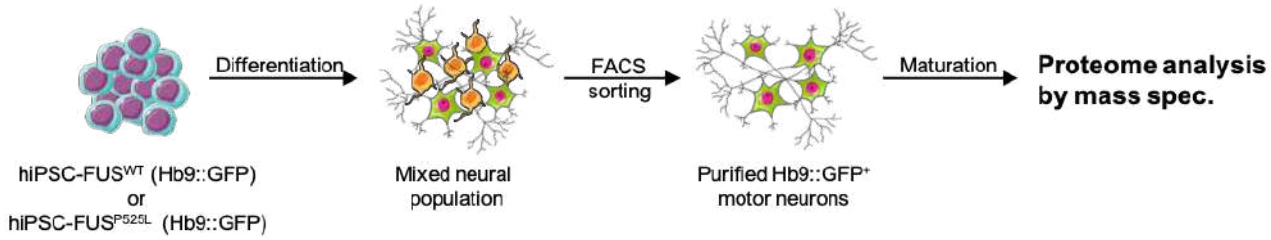
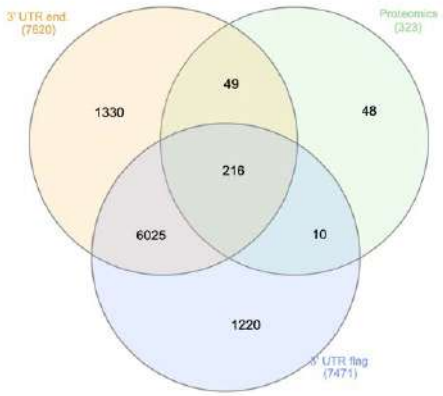


Figure 3. Outline of the proteome analysis by high-resolution LC-MS/MS performed in collaboration with A. Armirotti (IIT). Among GO terms relative to "biological processes" significantly enriched in the downregulated group of proteins, we found categories related to neuron development, differentiation and morphogenesis, and in particular to neuron projection (e.g. "neuron development", "axonogenesis", "neuron projection development"). Moreover, we found many terms relative to the cytoskeleton among cellular components.

By crossing proteomics (from the current project), transcriptomics and interactomics (from previous work) data, we gained insights into the molecular mechanisms underlying deregulation of cytoskeletal proteins. In particular, differential binding of mutant FUS to mRNA 3'UTR correlates with altered protein levels (Fig. 4). Interestingly, a number of genes encoding for upregulated or downregulated proteins, and direct targets of mutant FUS in the 3'UTR, had been previously linked to ALS, frontotemporal dementia and other neurodegenerative diseases. We are currently performing validation of the effects on translation of mutant FUS by a reporter system in which the luciferase coding sequence is fused to the target 3'UTR of selected candidates.

Figure 4. We interrogated the dataset that we have recently generated by PAR-CLIP analysis (De Santis et al., Cell Reports 2019; doi:10.1016/j.celrep.2019.05.085). Specifically, in that work we reported the fraction of the transcriptome bound by both endogenous and FLAG-tagged transgenic FUS, finding that wild-type FUS preferentially binds target transcripts in intronic regions, while FUS^{P525L} is mostly associated with 3'UTRs. Notably, a large fraction of altered proteins is encoded by genes bound by mutant FUS only in the 3'UTR (216/323, 66,9%). We performed GO term enrichment analysis of this latter group of genes and noticed terms relative to "RNA binding" and "Protein binding" molecular functions for both upregulated and downregulated proteins



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TREATMENT OF CHRONIC MYELOID LEUKEMIA BY INHIBITION OF TUBULIN POLYMERIZATION

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The research project aims to evaluate the therapeutic potential of tubulin polymerization inhibitors in the treatment of chronic myeloid leukemia (CML).

Despite the recent progress achieved through the use of tyrosine kinase inhibitors (TKIs), some CML patients are still resistant to this therapeutic intervention. The research aims to optimize the pharmacodynamic and pharmacokinetic properties of 3-aroyl-1,4-diarylpyrroles (ARDAPs), a new class of tubulin polymerization inhibitors, in order to identify one or more lead compounds with a potent anti-leukemic activity and improved pharmacokinetic characteristics for the treatment of those CML patients, that are resistant to modern TKIs.

We designed and synthesized three new series of ARDAP derivatives (68 compounds) in order to explore structure-activity relationships of the phenyl rings at position 1 (ring A) and 4 (ring B) of the pyrrole nucleus.

Among ARDAPs bearing a substituted phenyl ring at position 1 of the pyrrole, the 1-(4-bromophenyl) derivative was the most potent inhibitor of tubulin polymerization with an IC_{50} of 0.66 μM and of the growth of the human MCF-7 nonmetastatic breast cancer epithelial cells with an IC_{50} of 20 nM. ARDAPs bearing a substituent at position 4 of the 1-phenyl ring were generally superior to the corresponding 3-substituted counterparts. Introduction of a substituent at position 2 of the 4-phenyl ring provided tubulin polymerization inhibitors with IC_{50} values at submicromolar concentrations. The most potent MCF-7 cell growth inhibition correlated with the presence of a nitro group at position 2 of the phenyl at position 4. Among ARDAPs bearing substituents at both 1- and 4-phenyl rings, introduction of one or two amino group(s) at position(s) 3,4 of the phenyl rings resulted generally in highly potent inhibitors in both biochemical and cellular assays; seven derivatives showed the most potent MCF-7 cell growth inhibitors with nanomolar IC_{50}s.

Molecular modelling simulations highlighted the following key contacts into the colchicine binding site of tubulin: (i) the trimethoxyphenyl ring establish polar contacts with the Cys241β side chain and hydrophobic interactions with Leu248β and Leu255β; (ii) the B ring is involved in hydrophobic contacts with the Lys352β side chain and π-

cation interactions with the Lys352 ε-nitrogen atom; (iii) the A ring form hydrophobic interactions with Met259β, Lys353β, Ala180α and Val181α. In addition, the position of the substituent of both the A and B rings does not affect the binding mode of the new ARDAPs.

Five new ARDAPs inhibited the KU812, LAMA84-S, LAMA84-R, KBM5-WT and KBM5-T315I leukemic cell lines at low nanomolar concentrations and were definitely superior to the reference second generation TKI nilotinib (NLT) as inhibitors of the imatinib-resistant LAMA84-R and KBM5-T315I cells. Intriguingly, one of the tested compounds was uniformly effective as an inhibitor of the CML cell lines independently on the molecular mechanisms underlying TKI resistance. Indeed, LAMA-84R cells express heightened levels of Bcr/Abl protein and mRNA compared to LAMA84, whereas KBM5-T315I cells express a mutation in the drug binding site vs KMB5-WT. Our results therefore imply less risk of cross-resistance of the same compound with TKI and provide the basis of alternative synergistic targets for combined therapeutic strategies in CML-resistant and -sensitive cells. Accordingly, we observed that the tested compound enhanced NLT-mediated cell death in both KBM5-WT and KBM5-T315I CML cells, with the NLT/ARDAP combination superior to NLT or ARDAP alone in increasing the percentage of cells in apoptosis.

Five ARDAPs produced a dose-dependent inhibition of cell viability in glioblastoma T98G, U87MG and U343MG cells, in colorectal HT29, HCT116, SW480 and SW620 cells, and in urinary bladder T24 cells yielding low nanomolar IC_{50} values.

In the T24 and ES-2 animal models, one ARDAP exhibited significant inhibition of cancer cell proliferation, *in vivo* tumorigenesis, and tumour angiogenesis.

In conclusion, new ARDAPs were potent inhibitors of tubulin polymerization. Among the new derivatives, some compounds showed strong and broad-spectrum anticancer activity in breast carcinoma, leukemia, glioblastoma, colorectal and urinary bladder cancer cells. Obtained results highlight that the introduction of amino groups on both the 1- and 4-phenyl rings of the ARDAP scaffold is an effective strategy to obtain new broad-spectrum anticancer agents. We identified two robust lead compounds for the design of a new class of anticancer agents active in different types of solid and haematological tumours. These findings prompted the synthesis of new ARDAP analogues.

Publications

Puxeddu M, Shen H, Bai R, Coluccia A, Nalli M, Mazzoccoli C, Da Pozzo E, Cavallini C, Martini C, Orlando V, Biagioni S, Mazzoni C, Coluccia AML, Hamel E, Liu T, Silvestri R, La Regina G. *Structure-activity relationship studies and in vitro and in vivo anticancer activity of novel 3-aroyl-1,4-diarylpyrroles against solid tumors and hematological malignancies.* Eur J Med Chem 2020;**185**: e111828, doi 10.1016/j.ejmech.2019.111828, IF 4.833.

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