University of Modena and Reggio Emilia

PhD COURSE OF CLINICAL AND EXPERIMENTAL MEDICINE

PhD DAY 2017

Abstracts

April 19

2:00 p.m., Lecture Room H1.1

Department of Biomedical, Metabolic and Neural Sciences

(287 Campi street, Modena)

Five years ago Alessandro passed away. He was a former professor of our PhD School. Just below is reproduced the obituary appeared in BMJ 2012, 334: e1101.

Alessandro Liberati

Campaigned for high quality evidence relevant to patients

Nicola Magrini, Richard Smith

Alessandro Liberati, health services researcher and founder of the Italian Cochrane Centre (b 1954; q 1978, University of Milan) died on 1 January 2012 from multiple myeloma.

With his splendid name, fiery red hair, utter disdain for Silvio Berlusconi, and passion for Internazionale (the left wing team for those who don't know soccer), Alessandro Liberati had the look and feel of an Italian revolutionary. But he was a gentle and convivial revolutionary, regularly hosting the "libertrophy," a weekend party of fun and games at his family home in Tuscany. At his funeral in the packed Romanesque Basilica of Santo Stefano in Bologna, his daughter Valeria read his last letter, in which he hoped that there would be a special libertrophy "characterised by high spirits and by the desire to be together."

Butterfly behaviour

Alessandro's revolutionary fervour had clear aims: improving the quality of evidence available to patients and their clinicians; ensuring, as he wrote in the *BMJ* in 2004, that "research results should be easily accessible to people who need to make decisions about their own health" (*BMJ* 2004;328:531, doi:10.1136/bmj.328.7438.531); and trying to encourage researchers to concentrate on research that mattered to patients not to their careers or to drug companies. "How far can we tolerate," he wrote, "the butterfly behaviour of researchers, moving onto the next flower well before the previous one has been fully exploited."

These were concerns of Alessandro's early in his career after graduating in medicine from the University of Milan in 1978, but they were given a poignant intensity when in 1997 he was diagnosed as having "monoclonal gammopathy of uncertain significance (MGUS)." Modern medicine, he wrote, seems to be "good at creating 'new diseases' without necessarily knowing how to cure them."

After working for four years as a clinician and at the Instituto Mario Negri he won a scholarship to the Harvard School of Public Health and worked with Tom Chalmers, one of the first to promote systematic reviews of medical evidence. Alessandro learnt his trade working on important systematic reviews of treatment of early breast cancer and the effectiveness of antibiotic prophylaxis in patients in intensive care.

When the call came from Iain Chalmers, and others at the *BMJ*, in 1992 to start the Cochrane Collaboration, the international effort to synthesise medical evidence, he was one of the first to respond, and he contributed to the initial meeting of the collaboration in Oxford in 1993. Some have observed that the highly rational processes of evidence based medicine have been embraced more enthusiastically in northern, protestant countries than in southern, catholic ones, but Alessandro ensured that the Italian Cochrane Centre, which he led, was one of the first to be active. When he organised one of the early Cochrane Colloquium in 1999 in the beautiful building in Rome "where the pope went to school," the Italian minister of health emphasised the importance of evidence based medicine. Alessandro had influence: "He was a precursor of social networks in public health," says Maurizio Bonati, head of the department of public health at the Istituto Mario Negri, Milan, "making researchers work and stay together."

The most relevant evidence

But he was not a fanatic for evidence based medicine, says his colleague Marina Davoli. He rejected "the simplistic dichotomy . . . between experimental and observational studies . . . and the concept of the 'evidence hierarchy' [which puts systematic reviews at the top and case studies at the bottom]." Instead of asking "What is the best type of evidence?" she continues, he

suggested asking, "What is the most relevant evidence [for this decision to be taken], and what is its quality?"

Alessandro was also in recent years concerned about fraud in medical research and the malignant influence of conflicts of interest. He feared that the well of research might be poisoned and worried that newspapers like the *New York Times* might be better than medical journals at exposing and exploring conflicts of interest.

When he developed MGUS, he looked for the best evidence available and found that published reports didn't help much. Small studies suggested that the risk of malignant transformation was between 7% and 19%. Should he be monitored? Should he be treated? There was little evidence, but he had fought the "excesses of medicalisation in oncology" and so opted for no treatment. In 2002 his condition progressed to myeloma, and he was treated with a bone marrow transplant in May 2003. Should he have a second transplant? There had been four trials, but much to his irritation they weren't yet published. He went on to a second one in September 2003, moving into territory where evidence had little to say, but early trials may have overstated the benefits of a double transplant.

Just a few weeks before he died Alessandro published a letter in the *Lancet* in which he looked at the 1384 studies of multiple myeloma available in July 2011 and discovered that only 10 had overall survival as a primary endpoint and that there were no head to head trials of different treatments (*Lancet* 2011;378:1777-8, doi:10.1016/S0140-6736(11)61772-8). In other words, few of the studies were answering the questions that matter most to patients. He concluded, "If we want more relevant information to become available, a new research governance strategy is needed."

In his final years he had the chance to put his philosophy of improving clinical research governance into practice as the manager of research in the Regional Agency for Health and Social Care in Emilia-Romagna. Roberto Grilli, head of the agency, describes how he developed a process for selecting research studies to be funded, "focused on assessing not only their methodological robustness but also their potential relevance to patients and health care delivery." Grilli observes that what made Alessandro a "special person" was that his considerable scientific skills were "always backed by good beliefs and values mixed up with civil passion."

Alessandro leaves a wife, Mariangela, and two daughters.

Cite this as: *BMJ* 2012;344:e1101

The International Doctorate School in Clinical and Experimental Medicine (CEM) is organized by the Department of Biomedical, Metabolic and Neural Sciences in collaboration with other Departments of the University of Modena and Reggio Emilia and is under the direction of Prof. Giuseppe Biagini.

The educational program and research opportunities are directed towards the acquisition of skills required for basic and clinical research at Universities, public or private Research Institutes, and Hospitals. A Faculty of internationally recognized professors is responsible for the educational activities and takes part into the organization of the doctoral program.

From 2014 (cycle XXIX) the PhD Course of "Clinical and Experimental Medicine" is organized in 3 curricula:

Medicinal and Pharmaceutical Sciences
Translational Medicine
Health Sciences

From cycle XXV to XXVIII The Doctorate School of "Clinical and Experimental Medicine" was organized in 5 curricula/thematic areas:

Oncology
Public Health
Cellular and Molecular Pathophysiology
Clinical, Genetic and Molecular Medicine
Surgery

Cycle XXX

Dr. Lucia Borsari

CEM Curriculum: Health sciences
Tutor: Prof. Marco Vinceti

JOINT ASSOCIATION OF MATERNAL SMOKING AND PREGESTATIONAL DIABETES ON ADVERSE PREGNANCY OUTCOMES: A POPULATION-BASED STUDY IN NORTHERN ITALY

Background

Maternal tobacco smoking and pregestational diabetes (PGD) have been recognized as risk factors for adverse pregnancy outcomes, including premature birth and congenital anomalies [1,2]. Pregestational diabetes represents the most common chronic condition complicating pregnancy and the number of pregnancies affected has been increasing, particularly due to the obesity epidemic and consequent increase in type 2 diabetes in younger women [3]. Likewise, despite the known risk, most women smoking at conception and continue to smoke. In Italy, epidemiologic studies and routinely collected health data show that tobacco smoking prevalence among pregnant population varies from 6.7% to 22.3% [4]. To date no population-based study has still investigated whether smoking and PGD together may have a joint effect on pregnancy adverse outcomes.

Objectives

The aim of my research project was to investigate whether maternal smoking and PGD have a joint effect on premature birth and congenital anomalies among an Italian pregnant women cohort.

Methods

Using hospital discharges, we identified all women with PGD residing and delivering in the Emilia-Romagna region (Northern Italy) in the period 2007-2010 and we randomly selected five control women without diabetes. Our study endpoints were preterm births and congenital anomalies. We estimated the relative risks (RR) with 95% confidence intervals (CI) of these outcomes associated to smoking or PGD, and the joint effects of these factors by computing the relative excess risk due to interaction (RERI). Analyses were performed in the overall study population and in a restricted subgroup validated through diabetes registers.

Results

The study included 992 women with PGD (10.5% smokers) and 4,788 controls (11.9% smokers). Overall, we detected 524 (9.1%) preterm births and 118 (2.1%) newborns with one or more congenital anomaly. Compared to the reference category (non-smoking women without PGD), non-smoking diabetic women had RR=2.41 (CI 1.93-3.02) for preterm births and RR=1.41 (CI 0.88-2.25) for congenital anomalies, while smoking diabetic women had RR 4.69 (CI 2.97-7.41) and 2.65 (CI 1.05-6.71), respectively. Joint effect between smoking and PGD emerged for both preterm births (RERI=2.39, CI 0.25-4.53) and congenital anomalies (RERI=0.92, CI -1.28-3.12). Smoking together with

type 1 diabetes had a RERI of 16.79 (CI -36.28-69.88) for preterm births and with type 2 diabetes of 32.94 (CI -91.15-157.03) for congenital anomalies.

Conclusions

From these analyses, it resulted that smoking pregnant women with PGD have a higher RR of congenital anomalies and preterm births than both women with neither risk factors and women with only one of the two, therefore providing evidence for a synergy by these two factors. Moreover, the increased risk related to the combination of smoking and PGD resulted higher than the sum of the single effects of the two risk factors, indicating their positive joint effect in raising risk of both congenital anomalies (RERI=0.92) and preterm births (RERI=2.39). The joint effect between smoking during pregnancy and PGD was still more evident in the analysis restricted to the subgroup validated through the provincial diabetes registers, with RERI 4.58 for congenital anomalies and 1.75 for preterm birth, despite the reduced statistical precision of the risk estimates in this population due to the limited sample size. In conclusion, our results suggest that there is a positive interaction between maternal diabetes and tobacco smoking during pregnancy in increasing the risk of preterm births and congenital anomalies. Considering these possible joint effects and the high prevalence of smoking and diabetes in pregnant women, further efforts should therefore implemented to improve consciousness among diabetic women in childbearing age and encouraging quitting smoking, in addition to keeping good glycemic level before and during pregnancy.

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Dr. Virginia Brighenti

CEM Curriculum: Pharmaceutical Sciences
Tutor: Dr. Federica Pellati

NEW METHODS FOR THE STUDY AND CHARACTERIZATION OF NATURAL PRODUCTS AS SOURCES OF BIOACTIVE COMPOUNDS

Background

The use of natural products as supplements or substitutes to conventional drugs has remarkably increased in the developed countries [1]. A complete definition of all their phytochemical constituents by means of advanced analytical techniques, such as *metabolite profiling* and *fingerprinting*, is needed to ensure their composition, reliability and safety [2]. In this context, my research activity in this third year of PhD was focused on one plant of pharmaceutical interest (*Cannabis sativa* L., hemp) and one of nutraceutical relevance (*Vaccinium mirtyllus* L., bilberry), due to the bioactivity of their components.

Hemp is a dioecious plant, which is gaining a renewed increasing interest, thanks to the biological activity of non-psychoactive cannabinoids [3]. From a medicinal point of view, cannabidiol (CBD) represents the most interesting compound, possessing a high anti-oxidant, anti-inflammatory and neuroprotective activity as well as anxiolytic and anticonvulsant properties [4]. Given the outstanding pharmaceutical profile of these compounds, the selection of a suitable technique for the preparation of extracts highly rich in these bioactive constituents plays a key role.

Bilberry is a spontaneous plant native to the mountain areas of both Northern and Central Europe. Anthocyanins represent the most abundant class of bioactive compounds present in bilberry fruit, conferring it several health-promoting properties [5]. However, the content of these secondary metabolites in food products can be affected by the preparation process, which makes the monitoring of the quali- and quantitative profile of these compounds a crucial point for their genuineness and quality assurance [5].

Objectives

The study on hemp was aimed at the assessment and application of an efficient and selective technique in order to obtain extracts with a high content of non-psychoactive cannabinoids from specific hemp varieties to be tested for their neuroprotective properties with *in vitro* assays.

As regards bilberry, this study was aimed at the determination of the anthocyanin profile of berries and food derivatives, in order to monitor their quality in terms of bioactive compounds. Another aim of this project was the evaluation of the *in vivo* antioxidant activity of bilberry anthocyanins, by using the *Caenorhabditis elegans* model.

Methods

In relation to the assessment of an efficient technique for the extraction of cannabinoids from hemp, different methods and solvents were tested and compared, including dynamic maceration,

ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE) and supercritical-fluid extraction (SFE) with carbon dioxide (CO_2). A RP-HPLC method with UV/DAD and ESI-MS detection, previously developed during the second year of this PhD, was applied for the metabolite profiling of hemp extracts, by taking advantage of the innovative fused-core technology.
The metabolite profiling of anthocyanins in bilberry fruit and food derivatives was performed by means of a RP-HPLC method, with both UV/DAD and ESI-MS detection. Prior to the analysis, anthocyanins were selectively extracted from the samples by means of dynamic maceration with acidified methanol as the extraction solvent.

Results

As regards hemp, dynamic maceration at room temperature with ethanol as the extraction solvent proved to be the best technique in terms of recovery of non-psychoactive cannabinoids from the inflorescences. The extraction method and the HPLC technique were completely validated to show compliance with international ICH guidelines [6] and then successfully applied to the characterization of different hemp samples. In addition, extracts from selected hemp varieties were prepared and submitted to *in vitro* biological assays in order to identify those with the higher neuroprotective activity.
Concerning the evaluation of the anthocyanin content of bilberry fruit and derived food products, fourteen anthocyanins were detected and quantified in all the samples considered in this study. Bilbery fruit extracts with a high content of these compounds were also prepared and they are under *in vivo* evaluation for their antioxidant capacity, in collaboration with the Area of "Nutrición y Bromatología", Departamento de Química Analítica, Nutrición y Bromatología, University of Salamanca (Spain). The assays on *C. elegans* will include the evaluation of phenotypical characteristics, lifespan and resistance to oxidative and thermal stress.

Conclusions

Both the work summarized above demonstrate the great importance of the development of suitable and reliable analytical techniques in the ambit of the research in natural products, including both plants and functional food. As a matter of fact, the optimization of an efficient extraction technique for non-psychoactive cannabinoids from hemp, in combination with the HPLC method previously developed, represents a useful tool for the recognition of hemp as a plant of pharmaceutical interest, in addition to the well-known medicinal *Cannabis*. On the other hand, the study of the composition and content of anthocyanins in bilberry fruit and food products is of great importance in the ambit of both the quality assurance and dietary intake of these biologically active natural products.

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Dr. Erica Franceschini

CEM Curriculum: Translational Medicine
Tutor: Prof. Cristina Mussini

IMMUNOLOGICAL PREDICTORS OF SUCCESSFUL AND SAFE ANTI-REJECTION THERAPY IN HIV+ PATIENTS WHO UNDERWENT LIVER TRANSPLANTATION

Background

The Liver and Multivisceral Transplant Centre of the Policlinico Modena Hospital started a solid organ transplant (SOT) program for HIV infected patients in 2001 and 48 HIV patients have been transplanted so far. In particular, 43 HIV infected patients underwent a liver transplant and 5 had a combined kidney-liver transplant. 16 HIV transplanted patients out of 48 have died so far.

The risk for infection in the transplant recipient is a function of the interplay among host susceptibility, exposure to an opportunistic microorganism, and the inherent virulence of a given microorganism. Following SOT, immune responses of the recipient are profoundly altered. Several factors as immunosuppressive agents, donor-recipient mismatch, graft rejection, viral re-activation, transplantation and surgical trauma influence this immunodeficiency. Immunosuppressive therapy causes a marked suppression of cell-mediated immune responses. The depth of the deficiency of cell-mediated responses is influenced by the number and the type of immunosuppressive agents used, the dose of corticosteroids and other immunosuppressive agents, the use or absence of antithymocyte globulin, and the degree of mismatch between donor and recipient. Humoral immunity is also frequently impaired in the post-transplantation setting because of the use of corticosteroids and the deficiency in the cell-mediated responses that are necessary for T-cell dependent humoral responses.

In HIV infected patients that undergo a SOT, physicians have to consider HIV infection as another important player in patient net state of immunosuppression and as an infection risk factor.

Nowadays infections still remain the leading cause of morbidity and mortality among SOT recipients. Thus, it is essential to understand which are the impairments in humoral and cell-mediated immunity after transplant, when such impairments are detectable, and which kind of immunity alterations can lead to a real and clinically significant increase in the risk of infections.

An immunological approach could help clinicians to classify transplanted patients in different infection risk categories, helping in differentiating prophylaxes and therapies on the basis of their infection risk.

Objectives

A successful anti-rejection treatment has, on one side, to block the immune response against the SOT, and, on the other, to allow an efficient immune response against pathogens. Thus, the main aim of my PhD program is to identify which characteristics of the specific immune response can predict the clinical success of such therapy. For this reason, in transplanted HIV-positive patients, transplanted HIV-negative patients, non-transplanted HIV-positive patients and controls (HIV-negative, non-transplanted patients), my PhD project focuses on:

- Comparison of the T cell response to a number of relevant and recall antigens, in terms of the identification of the quantity and of the quality (i.e., number of functions simultaneously performed by a single cell) of the specific response;
- Analysis of molecular and cellular parameters related to the activation or inhibition of either the innate or adaptive response, paying a particular attention for the mechanisms involved in the triggering of inflammation and in the activity of the inflammasome system.

The secondary aim of the project is to find an immunological predictor of vulnerability to infection, that has a large applicability and is able to help clinicians in stratifying the infection risk of transplanted patients, either HIV-positive or negative.

Methods

Case-control single-centre study.

We are evaluating the aforementioned immune parameters in all alive HIV-positive patients who have received a SOT at the Liver and Multivisceral Transplant Centre (Policlinico Hospital, Modena) from 2001 to 2016. We are evaluating the same T-cell responses in other three groups (transplanted HIV-negative patients, non-transplanted HIV-positive patients, and HIV-negative, non-transplanted controls).

Patients are matched for age, sex, MELD, year of transplant, immunosuppressive regimen, absolute CD4 cell number, and antiretroviral treatment.

The study was approved by the Ethical Committee (protocol number 163/15, 23th October, 2015). Each participant provided written informed consent for testing and analysis of samples.

All of the immunological studies are performed in collaboration with the Chair of Pathology and Immunology (Prof. Andrea Cossarizza).

We collected 30 ml of peripheral blood for each patient. Plasma was stored at -80°C until use, mononuclear cells (PBMC) were isolated according to standardized methods and immediately used.

Freshly isolated PBMC were stimulated with PepMix BKV (Large T antigen), PepMIX *Candida albicans* (MP65), PepMix CEFT Pool, PepMix HCMVA (UL32), PepMix VACV (MVA 074R), all from JPT (Berlin, Germany), *Staphylococcus aureus* enterotoxin B (SEB) and anti-CD3/CD28/CD49d for 16 hours. After *in vitro* stimulation, cells were stained with AQUA Live/Dead, and anti-CD3 PE-Cy5, -CD4 AF700, -CD8 APC-Cy7, -CD45RA PE, -CCR7 BV421 mAbs (Biolegend, CA-USA). Cells were fixed and permeabilized with Fixation/Permeabilization Solution Kit (Becton Dickinson, CA-USA). Cytokine production was assessed by intracellular staining by using mAbs directly conjugated recognizing IL-2-APC, IL-17-PE-Cy7, TNF-BV605, IFN-γ-FITC (all from Biolegend). Finally, cells were acquired by using Attune NxT (Thermo Fisher, USA). A minimum of 5 million cells per sample were acquired.

The other part of PBMC will be used for molecular analysis. In particular, monocytes will be isolated by magnetic sorting using an anti-CD14 mAb; then RNA will be extracted for the real time PCR analysis (by using a CFX96, BioRad) and quantification of genes involved in the activation and regulation of the inflammasome, such as NLRP3, AIM2, NLRC4, NLRX1.

Results

Until now, 9 HIV+ patients who underwent LT and 9 age- and sex-matched healthy subjects (CTR) were enrolled. All patients but one took tacrolimus as anti-rejection treatment, one had cyclosporine. Seven patients out of 9 had highly active antiretroviral therapy containing integrase inhibitors, the other two ELV/COBI/TDF/FTC. All patients but one were HCV-positive before LT but all of them obtained a sustained virological response at the time of the analysis. The median CD4 count was 538.5 cells/uL (range 207-665) and all patients had undetectable HIV-viral load. All transplantations were performed more than one year before the study, and no infective events were present at enrolment.

The percentage of CD4+ T cells in LT patients was decreased if compared to CTR, while CD8+ T cells were increased. LT and CTR had a similar distribution of different T cell subsets (naïve, central memory, effector memory). In both groups, and in a similar manner, cytokine production after 16 hours of *in vitro* stimulation with recall antigens was scanty, while a higher response was observed after stimulation with SEB or anti-CD3/CD28/CD49d. The percentage of CD8+ T cells producing TNF-α and IFN-γ was much higher in LT patients if compared to CTR.

Conclusions

Cells from patients with severe immune alterations due either to HIV infection or anti-rejection therapy are still capable of responding to external stimuli. Taking into account that no patient had clinically relevant infections in the three-month period before enrollment, it is tempting to speculate that an adequate control of immune activation, that can be easily monitored by flow cytometry, not only can inhibit the rejection of the transplant, but also to avoid infection development.

Dr. Lavinia Giva

CEM Curriculum: Translational Medicine
Tutor: Prof. Manuela Simoni
CoTutor: Dr. Francesco Potì

SPHINGOSINE-1-PHOSPHATE (S1P) AND ATHEROSCLEROSIS: NEW EVIDENCE ON THE TRANSGENIC MOUSE MODELS

Background

S1P is a lysosphingolipid which regulates many important biological functions, such as cellular proliferation, survival and differentiation, through the interaction with five specific sphingosine 1-phosphate receptors (S1PRs) belonging to the G-protein coupled receptor superfamily. In plasma, S1P is associated with the high density lipoproteins (HDL), and several studies documented an inverse relationship between HDL cholesterol levels and the extent of atherosclerotic disease. These recent findings suggest that the HDL atheroprotective effects could be partially attributed to S1P, in particular through the stimulation of S1PR1/3, on vascular wall cells (macrophages and endothelial cells).

Objectives

Therefore, our project was focused on clarifying the effects of endogenous S1P on atherosclerosis in vivo, generating worldwide unique mouse models able to overexpress S1PR1 or S1PR3 in specific target tissues (S1P1-Lyz and S1P3-Lyz transgenic mice).

Methods

We obtained knock-in mouse models, based on the Cre-Lox technology, to amplify the endogenous S1P signaling through the overexpression of S1P1 or S1P3 receptors in specific target tissues (macrophages and endothelial cells). Since the S1P1-Lyz and S1P3-Lyz mouse models are on C57BL6 background which is resistant to atherosclerosis development, we currently crossbreeded S1P1-Lyz and S1P3-Lyz mice with LDLR-/- mice to generate athero-prone strains. These mice were fed a HFD for 16-24 weeks. We collected and stained (Oil Red O / Hematoxylin staining) heart cryosections, in particular aortic roots and brachiocephalic artery, in order to analyze atherosclerosis expressed as the total area of the lesion or the ratio between the area of the plaque and the total area of the vessel.

Results

Animals overexpressing S1PR1 in macrophages showed a massive reduction of atherosclerotic lesions versus controls, both in aortic root sections and in brachiocephalic artery ones. Similarly in the endothelium, the overexpression of S1PR1/3 induced a significant reduction of the lesions versus controls.

Conclusions

In the present work, we demonstrated for the first time that the amplification of endogenous S1P signaling revealed an atheroprotective role and its positive effects could be partially associated with both S1PR1 and S1PR3 receptor.

Dr. Maurizio Greco

CEM Curriculum: Translational Medicine
Tutor: Prof. Giovanni Pellacani

THE DIFFERENT WAYS SKIN REACTS DUE TO DIFFERENT SOURCES OF DAMAGE: MORPHOLOGICAL STUDY IN VIVO AND BIOLOGICAL CHARACTERIZATION OF TISSUE

Background

The mechanism of tissue changes after laser therapy is not still completely clear. A review of literature shows only data about histopatological changes after laser treatment. To obtain this information, several skin biopsies were performed, often on face or other sensitive areas. The main limitation is the inevitable scar formation after the biopsies.

Usually, lasers are considered safe treatments with a rapid healing time. Post-treatment adverse effects may occurs, such as pigmentation disorders. However, at my best knowledge, there are not consistent data concerning long term effects of lasers, in particular on their potential carcinogenic effects.

Objectives

The main purpose of this study is to explore laser effects in different skin conditions and to highlight the biological effects on different tissues in order to evaluate potential carcinogenic effects of different wavelengths and energies, and to discover the optimal light source to target a specific tissue or a disease.

Methods

My research interest is to analyze laser-tissue interactions and biologic effects of various laser sources by means of in vivo Reflectance Confocal Microscopy (RCM) and Optical Coherence Tomography (OCT). Many skin diseases (non-melanoma skin cancer, melasma, sarcoidosis, keloids, etc.) could be treated with different laser sources, in particular CO2, fraxel CO2, flash dye pump laser, NdYag 532/1064 and CW 532/1064. Whereas distinct lasers interact differently with tissues, my purpose is to investigate the effects both in ex-vivo cell cultures and in in-vivo settings.

RCM is a non-invasive imaging technique that allows to acquire skin images with a histological resolutions.

OCT is a quite novel technique that uses infrared light emission to study the architecture of the skin in depth.

First study - Combination of laser CO2 with photodynamic therapy (PDT) in the treatment of basal cell carcinoma (BCC)

Two patients with histological diagnosis of nodular BCC and two with histological diagnosis of infiltrative BCC of the face, have been treated with this combined modality. We performed a long-term follow-up using clinical and dermoscopic documentation and RCM images.

Second study – Efficacy of fractional ablative CO2 laser therapy for striae distensae.

18 patients (16 women and 2 men) with striae distensae were divided in two group and treated for six months with a different number of sessions of fractional CO2 laser and with a different power. Improvement has been evaluated by comparing pre- and post-treatment clinical pictures and RCM images. This study has established the efficacy and safety of fractional CO2 laser therapy for striae distensae.

Third study – Efficacy of fractional ablative CO2 laser and Flash Dye Pump laser and Corticosteroid infiltration for the treatment of keloids

Seven patients with keloids were treated once a month for three months. Clinical images and OCT images were acquired before and after the end of treatments. This study has established the efficacy of this combined therapy for keloid.

Results

The results of these studies would show the use of laser therapy as a choice in both inflammatory and tumoral skin diseases, in particular for those cases that can't be treated efficaciously with other treatments. Furthermore, these techiniques have a high safety profile. The outcome is to improve the knowledge about this topic and to offer less invasive and safer treatments for the patients.

First study - Combination of laser CO2 with photodynamic therapy (PDT) in the treatment of basal cell carcinoma (BCC)

Preliminary results have shown that the mean age is 48 years with a range of 32 to 80. Patients are still in follow-up (3 years). Only one patient had a recurrence (infiltrative BCC) after 6 months of follow-up, it was performed the same therapy again and he is still in complete remission. No significant complications were observed.

Second study – Efficacy of fractional ablative CO2 laser therapy for striae distensae.

Clinical improvement is visible by pictures comparing. RCM images have shown significant changes after the therapy, in particular the progressive improvement of the collagen in the dermis and the restoration of the dermal-epidermal junction architecture. In any case, worsening of striae distensae has been detected.

Third study – Efficacy of fractional ablative CO2 laser and Flash Dye Pump laser and Corticosteroid infiltration for the treatment of keloids

Clinical improvement is visible by pictures comparing. OCT images show a decrease of thickness and vascularization. An overall improvement has been reported from all he patients, in particular the disappearance of itchy.

Conclusions

First study - Combination of laser CO2 with photodynamic therapy (PDT) in the treatment of basal cell carcinoma (BCC)

This combination of laser and PDT led to complete recovery of the diseases (BCC nodular and infiltrative) and, moreover, a better aesthetic outcome than surgery.

Second study – Efficacy of fractional ablative CO2 laser therapy for striae distensae

These results support the use of ablative CO2 fractional laser as an effective and safe modalities to treat striae distensae.

Third study – Efficacy of fractional ablative CO2 laser and Flash Dye Pump laser and Corticosteroid infiltration for the treatment of keloids

These results support the use of a combine therapy for the treatment of keloids. In particular fractional CO2 laser and corticosteroids infiltration act on the hardness and the thickness of keloid, flash dye pump laser instead acts on the vascular component of the lesion.

Dr. Eleonora Maretti

CEM Curriculum: Medicinal and Pharmaceutical Sciences
Tutor: Prof. Eliana Grazia Leo
CoTutor: Dr. Valentina Iannuccelli

SURFACE ENGINEERING OF SOLID LIPID NANOPARTICLE ASSEMBLIES BY METHYL α-D-MANNOPYRANOSIDE FOR THE ACTIVE TARGETING TO MACROPHAGES IN ANTI-TB INHALATION THERAPY

Background

Tuberculosis (TB) is caused by *Mycobacterium tuberculosis* (Mtb), which may attack mainly the lungs. Pulmonary route appears the most promising way to reach promptly the infected site. Two basic requirements should be fulfilled in the design of inhaled formulations to achieve effective drug delivery inside alveolar macrophages (AM). Firstly, drugs should be able to reach AM after administration. Secondly, drugs should be taken up by AM. The passive targeting to the alveoli entails the development of particles with some essential properties: narrow aerodynamic diameter range (0.5 – 5 μm), negative surface charge, irregular morphology, and high physico-chemical stability. In this regard, microparticles have been demonstrated to deliver a high payload of drugs and to be efficiently phagocytozed by AM. One of the advantages using these formulations concerns the possibility to insert at the surface of the particles specific molecules that recognize receptors on the target site. Mannose receptors (MR), overexpressed in infected AM, can recognize carriers bearing surface-exposed mannose residues and facilitate their internalization. We previously developed assemblies of Solid Lipid Nanoparticles (SLNas) for an inhaled anti-TB therapy by Dry Powder Inhaler (DPI) devices as a carrier of rifampicin (RIF), a first-line anti-TB drug. The designed SLNas were found to be a useful carrier for RIF and provide effective AM passive targeting [1,2].

Objectives

The present research explores the specific targeting of a clinically used anti-TB drug by means of surface engineered SLNas directed to AM by pulmonary route. The success of these carriers, known to be biocompatible and biodegradable, is based on a careful selection of materials as well as an evaluation of the particle physical characteristics and surface features. The purpose of this work was to explore and to maximize the phagocytosis process of mannosylated SLNas developed by using a novel and manageable functionalization technique as well as to study the effect of the surface modification on SLNas aerodynamic performances.

Methods

SLNas were prepared employing the melt emulsification technique followed by freeze-drying. Two sample sets were obtained by using cholesteryl myristate mixed with palmitic acid (PA set) or tripalmitin (TP set) as the lipid phase. These lipid components were processed in the presence of sodium taurocholate (ST) as the biocompatible stabilizer and methyl α-D-mannopyranoside (MP) as

the functionalizing agent obtaining two SLNas sets composing of different drug/lipid ratios and drug solubilization method. The obtained mannosylated SLNas were examined for their physico-chemical properties: morphology by Transmission Electron Microscopy, particle size, surface charge, and polydispersity index by Photon Correlation Spectroscopy, physical state of the components by Differential Scanning Calorimetry, drug loading and release. The optimized samples were investigated for the presence of mannose residues on the particle surface in comparison with the same samples without mannose, named non-functionalized SLNas (NF-SLNas). A complementary suite of surface techniques such as Fourier Transform Infra-Red spectroscopic analysis, elemental composition by Energy Dispersive X-ray spectroscopy, X-ray Photoelectron Spectroscopy, and wettability was used. Lastly, cytotoxicity, ability to be taken up by J774 macrophage cell line through MTT test and flow cytometry/confocal microscopy, respectively, as well as *in vitro* aerodynamic performance by Next Generation Impactor (NGI) were tested.

Results

SLNas appear irregular with a main population sized around 1 µm. The irregular particle shape plays a relevant role in powder aerodynamic performance, providing a better powder de-aggregation and fluidization capacity due to the less particle contact area. Moreover, the SLNas negative surface charge is a favorable property giving lower cytotoxicity compared to positive or neutral particles and promoting the internalization process by AM. Besides the suitable physical properties, SLNas displayed sufficient drug payload (from 10 to 15%) to achieve a therapeutic effect in function of a feasible administration dose inside DPI device and negligible drug spreading over the lung fluid before AM uptake. Although all the samples were found to be appropriate concerning the physical characteristics, two of them exhibiting the highest encapsulation efficiency and drug loading levels were investigated for mannose surface engineering in comparison with NF-SLNas samples. All the results of the analyses performed contribute to support the hypothesis that the mannose derivative remained lipid associated reasonably owing to the hydrophobic interactions between MP and the lipid matrix. Unlike TP and NF-TP samples, PA and NF-PA were found to be non-cytotoxic probably due to the lesser amount of aggregates. PA sample was taken up quickly by macrophages, reaching after 15 min the intracellular fluorescence plateau until the end of the experiment. On the contrary, a negligible fluorescence was recorded from NF-PA at both 15 and 30 min. Although these successful results, the surface functionalization influenced negatively the aerodynamic performance in terms of a massive reduction in respirability [4].

Conclusions

Despite favorable physical properties, respirability was impaired by the powders cohesiveness of functionalized SLNas. Concerning the active targeting, the presence of MP on SLNas surface caused an improvement of the internalization rate by AM. Owing to the proven effectiveness of mannose in AM uptake process, more balanced amphiphiles bearing α-D-mannose residues that could lead to a different arrangement of the targeting moiety on SLNas surface will be considered for further studies.

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THE EXTENT OF WHOLE-GENOME COPY NUMBER ALTERATIONS PREDICTS AGGRESSIVE FEATURES IN PRIMARY MELANOMAS

Background
Cutaneous melanoma is the most aggressive form of skin cancer and encompasses a heterogeneous family of tumors that differ in terms of clinical aspects and biologic behavior, ranging from indolent tumors with a slow growth rate, very low aggressive potential and good prognosis, to highly aggressive tumors with a fast growth rate, high metastatic potential and possible fatal outcome. Recent suggests that specific morphological features may help predict its clinical behavior**.**

Objectives
The Scope of the project was to explore the genetic heterogeneity and the relationship between genetic background and clinical–morphological features in primary melanoma.

Methods
Using a SNP-array approach, we quantified chromosomal copy number alterations (CNA) across the whole genome in 41 primary melanomas and found a high degree of heterogeneity in their genomic asset. Association analysis correlating the number and relative length of CNA with clinical, morphological, and dermoscopic attributes of melanoma was performed.

Results
Using a SNP-array approach, we found a high degree of heterogeneity in their genomic asset. Association analysis correlating the number and relative length of CNA with clinical, morphological, and dermoscopic attributes of melanoma revealed that features of aggressiveness were strongly linked to the overall amount of genomic damage. Furthermore, we observed that melanoma progression and survival were mainly affected by a low number of large chromosome losses and a high number of small gains. We identified the alterations most frequently associated with aggressive melanoma, and by integrating our data with publicly available gene expression profiles, we identified five genes which expression was found to be necessary for melanoma cells proliferation

Conclusions
In conclusion, this work provides new evidence that the phenotypic heterogeneity of melanoma reflects a parallel genetic diversity and lays the basis to define novel strategies for a more precise prognostic stratification of patients.

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THE SYNTHETIC KILLER PEPTIDE IMPAIRS CANDIDA ALBICANS BIOFILM FORMATION AND PERSISTENCE

Background

Candida albicans colonizes human skin and mucosae of healthy individuals, behaving as a harmless commensal. Nevertheless, in susceptible patients (i.e., subjects with medical devices or immunosuppressed individuals), Candida behaves as an opportunistic pathogen also because of its ability to produce biofilms on mucosal and abiotic surfaces. Extensive literature describes biofilm production as a critical virulence factor, through which many microorganisms, including C. albicans, enhance their pathogenic potential. In particular, when embedded in a biofilm, Candida becomes more resistant to common disinfectants and antibiotic treatments. From here, the demand of novel therapeutic approaches against C. albicans biofilm.

Recently, several antibody-derived peptides have been shown to have antimicrobial, antiviral, immunomodulatory and antitumor activity both in vitro and in vivo. Killer peptide (KP) is the first engineered decapeptide (AKVTMTCSAS) which is able to maintain the anti-microbial activity of its native antibody via interaction with specific receptors on microbial target. Notably, KP is effective against several fungal pathogens, including C. albicans, irrespective of their resistance to conventional antifungal agents (1). To date, no information is available on the possible KP effect against C. albicans biofilm.

Objectives

The aim of the project is to evaluate the effect of KP, on the formation and persistence of C. albicans biofilm. Briefly, a laboratory strain and several clinical fungal isolates have been treated with KP and the following parameters were evaluated:

- production/persistence of biofilm;
- production of reactive oxygen species and O_2^-;
- transcriptional profiles of biofilm-associated genes;
- biofilm formation and persistence on central venous catheter (CVC).

Methods

The reference strain SC5314, two wild type clinical isolates (DSY544 and DSY347) and two clinical isolates which had been knocked out for their resistance mechanisms to fluconazole (DSY775 was derived from DSY544; DSY289 was derived from DSY347), were employed in the study (2). In selected experiments, also the bioluminescent C. albicans SC5314, transformed with CIp10::ACT1p-gLUC59 plasmid, was used (3).

KP was tested in parallel with the scramble peptide (SP) containing the same 10 aminoacids of KP allocated in a different sequence. The latter was used as a negative control.

The effects of KP and SP were assessed against Candida biofilm at different stages of development, by crystal violet and tetrazolium salt reduction assays (to assess total biomass and residual metabolic activity, respectively). Moreover, KP-induced reactive oxygen species (ROS) production (commercially available kits) and the effects on the transcription levels (qRT-PCR) of genes related to cell adhesion, hyphal development and extracellular matrix production were investigated. Finally, KP capability to interfere with C. albicans biofilm on a CVC was assessed.

Results

KP affected biofilm in terms of both total mass and metabolic activity, in a dose-dependent manner. The exposure of C. albicans biofilm to KP enhanced ROS production as well as the number of PI fluorescent cells, indicative of a reduced viability. Moreover, almost all gene transcript levels were down-regulated by KP treatment, both when given at early stages of biofilm formation or added to mature biofilm. Notably, similar inhibitory effects were observed against all the C. albicans strains employed, irrespective of their resistance or susceptibility to fluconazole.

Finally, KP treatment significantly inhibited biofilm formation and persistence on CVC, as demonstrated by i) the decrease in relative luminescent units emitted by live Candida cells and ii) the reduction of viable CFU.

No effects were ever observed when using SP as negative control, irrespective of the assay employed.

Conclusions

This pilot in vitro study represents the first report describing the anti-biofilm activity of KP, comparably evident against resistant and susceptible isolates. This implies that KP might be considered as a novel therapeutic tool against biofilm-associated infections, efficacious also independently of the drug-resistance fungal phenotypes.

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COMPUTATIONAL APPROACHES IN POLYPHARMACOLOGY

Introduction

Traditional drug discovery aims at identify a chemical entity, which selectively modulates the activity of a target of interest. Nevertheless, recent advancements in understanding protein pharmacology have led to an increasing evidence that drugs are not highly selective, but rather interact with multiple targets at the same time. This phenomenon, known as polypharmacology, is recognized to be as an opportunity for future drug discovery.[1] In fact, it allows the rational development of a unique chemical entity that hits a selected pool of targets, potentially avoiding unwanted drug–drug interactions, resistance or side effects.[2] In this regard, computational approaches can offer the possibility to predict the activity profile of ligands to a set of targets, therefore distinguishing between beneficial (synergy) from unwanted (side-effect) target affinities. Here, two explorations of how *in silico* approaches can be applied in the effort of multi-target drug design are presented: an *in silico* polypharmacological analysis of structural databases, and an application of *in silico* strategies to design challenging Hsp90- dual inhibitors.

PREDICTING DRUG POLYPHARMACOLOGY USING STRUCTURAL DATABASES

Background

The Protein Data Bank (PDB)[3] is a well-known, huge and ever-growing reservoir of structural and biological information to use, either as starting point in an *in silico* driven drug discovery process and to highlight and/or solve selectivity issues. More than 128,000 biological macromolecular structures obtained from x-ray crystallography, solution and solid state NMR, neutron diffraction and electron crystallography are housed into the PDB database (accessed on: 27/03/2016). However, only a limited portion of all of this information has been exploited to prospectively design “truly” selective compounds while performing *in silico* drug design.

Objectives

In an effort to identifying new multi-target activity profiles of compounds deposited into the PDB, the structures of biological targets in complex with their ligands were sistematically analyzed by integrating ligand-based and structure-based *in silico* approaches.

Methods

First, all crystal structures of human proteins were extracted from the PDB database (accessed on: April 6, 2015) and prepared for the subsequent calculations using the Protein Preparation Wizard utility available in Maestro 10.3.[4,5] Then, ligands were extracted from the complexes in their native poses. The generated database of ligands was filtered to remove solvent molecules, fragments, peptides and compounds with predicted unfavorable pharmacokinetic properties.[6,7] Afterwards, an *all-against-all* 3D ligand-based virtual screening was performed to evaluate the profile of similarities of each ligand in the pretreated database. Pairs of compounds for which a high degree of similarity was evaluated were further inspected by cross-docking the ligands into the binding site of the crystal structures.[8] A close visual inspection of the complexes was made to assess the presence of key ligand-protein interaction across different targets. Finally, the activity profile of compounds showing a high degree of similarity and a well-defined ligand-protein complementarity in the complexes were checked in the literature.

Results

As described in the previous section, by integrating structure-based and ligand-based virtual screening approaches, similarities between co-crystallized ligands and ligand-protein complementarities were established. Remarkably, the combination of these two approaches has provided more robust results and helped in overcoming intrinsic limitations derived by peculiar features of the methods. These analyses allowed to depict several potentially relevant ligand-target associations. Some of them were already known in the literature, providing an internal validation to the applied method. Many others are still unknown and their potential activity for the predicted biological targets will be evaluated prospectively.

Conclusions

The design of a chemical entity that simultaneously and selectively modulates a selected pool of targets represents an attracting goal, especially for the treatment of complex diseases.[2] One key strength of analyzing the PDB database is the possibility to know and exploit the structural details in a protein-ligand complex that are crucial for binding. The obtained results suggests new insights into the relationships between protein targets and ligands deposited in the PDB. Therefore, helping to disclose the “true” selectivity of already known compounds and, to design novel selective multi-target modulators.

***IN SILICO* STRATEGIES TO DESIGN CHALLENGING HSP90- DUAL INHIBITORS**

Background

Hsp90 is a chaperone protein highly conserved from bacterial to mammal organisms. In non-cancerous cells, Hsp90 activity is indirectly involved in the regulation of many cellular processes, whereas it has been found to be up-regulated in several cancers supporting its implication in tumorigenesis and in drug resistance mechanisms.[9] Hsp90 has represented a promising drug target to develop small molecule inhibitors for a long time. Nevertheless, no drugs targeting Hsp90 have been approved so far, while Hsp90 inhibitors are highly explored in combination therapies.[10] It is well known that complex diseases such cancers usually require the modulation of more targets at the same time due to incomplete responses or induced resistance mechanisms.[11] Interestingly, many members of the Hsp90 interactome are validated anticancer drug targets. Among them, B-Raf and HDAC6 were selected as promising candidates for the development of multi-target inhibitors. In fact, these two targets play a pivotal role in the development of diverse types of cancer for which responses to the pharmacological treatment are rarely complete. In details, despite RAF inhibitors have been recently approved to treat patients with the BRAF-V600E mutant melanoma, resistance mechanisms still lead to unsatisfying responses. Remarkably, the combination of Hsp90 inhibitors with B-Raf inhibitors showed significant synergistic effects and the resulting drug combinations is currently being evaluated in clinical trials.[12,13] HDAC6 has gained significant attention in drug discovery because of its implication in many cancer-relevant processes and the positive outcome in patients.[14] However, the efficacy of HDAC6 inhibitors as single-agent therapy may be still limited. Notably, the combination of HDAC6 inhibitors and Hsp90 inhibitors exhibited synergistic cytotoxic effects.[15] In the light of the previous considerations, the development of such Hsp90- dual inhibitors represent a valuable strategy to design compounds with improved therapeutic effects.

Objectives

Here, the application of two tailored *in silico* strategies devised to design Hsp90/B-Raf and Hsp90/HDAC6 dual inhibitors are presented.

Methods

- *Identification of Hsp90/B-Raf dual inhibitors*

First, databases of commercially available compounds were filtered in order to remove ligands with unfavorable pharmacokinetic profiles.[7] Then, filtered databases were screened against a pharmacophore model based on shared features of co-crystallized ligands of both targets. Afterwards, the resulting libraries were docked in the crystal structures of BRAF and Hsp90 in order to identify compounds showing a high degree of ligand-protein complementarity for both targets. A final step of visual inspection was attempted to select compounds for the bioassays. Furthermore, control calculations were performed by 2D fingerprint and 3D shape-based analyses to assess the presence of common chemical patterns between the scaffolds of selected ligands and known inhibitors of Hsp90 and B-Raf reported in the ChEMBL database.[16]

- *Identification of Hsp90/HDAC6 dual inhibitors*

HDAC6 inhibitors were collected from the ChEMBL database (accessed on January 10 2017) and filtered based on the enzyme assay outcome type.[16] Starting from this set of active ligands, common shape query hypotheses were built.[17] Afterwards, using an *in house* developed python script, databases collected from different vendors were filtered removing compounds not presenting reported Zing Binding Domain (ZBD) groups.[14] Then, the pre-filtered databases were screened on the shape queries to remove compounds with a scaffold distant to that of known HDAC6 selective inhibitors.[17] Finally, the resulting databases were docked into HDAC6 and Hsp90 *crystal structures* previously prepared. A visual inspection of the complexes and an analysis of scores led to a final selection of compounds for the bioassays.

Results

According to the workflows described in the previous section, several compounds were selected to be assayed. In case of the Hsp90/B-Raf dual inhibitors design, the first-in-class multi-target inhibitor was disclosed through a tailored *in silico* strategy. The newly identified compounds show low molecular weights and a balanced *in vitro* potency for the two targets therefore, represent promising starting points for hit-to-lead optimization phases. The results have been submitted for publication. In case of the Hsp90/HDAC6 dual inhibitors design, different vendor databases were screened to recover ligand presenting structural features crucial for the binding in HDAC6 and Hsp90 targets. Most interesting compounds with novel scaffolds and favourable interactions on both the targets were selected and are currently being experimentally assayed.

Conclusions

Despite the simultaneous inhibition of B-Raf and Hsp90 or Hsp90 and HDAC6 seems to provides a clear benefit on pharmacological treatment of various types of cancers, to the best of our knowledge, no B-Raf/Hsp90 or Hsp90/HDAC6 dual inhibitors have been identified so far. Our research findings suggest that the application of a tailored *in silico* strategy could help in identifying such challenging dual inhibitors, as demonstrated by the results obtained in the Hsp90/B-Raf dual inhibitors design.

For HDAC6/Hsp90 dual inhibitors design, a rigorous *in silico* strategy especially devised for the identification multi-target ligands has led to a section of set of compounds that are being experimentally assayed. If active compounds are identified, further structure activity relationship studies will be carried out for most promising scaffolds.

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SYMPTOMS ASSESSED BY A SIMPLE, SELF-ADMINISTERED QUESTIONNAIRE MAY GUIDE IN DIFFERENTIATING DYSPNEIC PATIENTS WITH COPD, HEART FAILURE OR BOTH

Background

The interaction between the lung and the heart is a complex, vast and fascinating subject, and, from a clinical point of view, disorders of one often influence and promote disorders of the other. This is especially true for chronic diseases, and COPD represents a classic example: the association between COPD and heart diseases, e.g. heart failure and coronary artery disease, has long been a matter of discussion; cardiac comorbidities in patients with COPD are the main research topic of our group[1][2]. COPD is a frequent, chronic respiratory disease occurring mostly in elderly smokers or ex-smokers. It is characterized by persistent respiratory symptoms and airflow limitation, which is due to airway or alveolar abnormalities usually caused by significant exposure to noxious particles or gases[3]. Dyspnea, sometimes associated with chronic cough and sputum, dominates the clinical presentation. Concomitant chronic cardiac disorders are frequent in patients with COPD, likely due to shared risk factors (e.g. ageing, smoking, inactivity, metabolic disorders, altered inflammatory response)[1]. Heart failure, a clinical syndrome caused by a structural and/or functional cardiac abnormality, is one of the most frequent and important cardiac comorbidity: prevalence estimates indicate that about one in four patients with COPD may have concomitant HF, which is often undiagnosed and untreated, and vice versa, COPD prevalence in HF is about 13-39%[4]. The coexistence of COPD and HF have long been a topic of discussion, given the challenges in making a correct diagnosis, and the significant impact on patient prognosis and quality of life [5].

The differential diagnosis of COPD in HF patients, and vice versa, is challenging due to substantial similarity in clinical presentation. Dyspnea, associated with reduced exercise tolerance, and fatigue are common in both diseases, and often the clinical picture does not allow a correct distinction between patients with COPD only, HF only, or both disorders[6]. The differential diagnosis is particularly difficult in the elderly subjects with chronic symptoms and a positive history of smoking exposure, which represent a substantial portion of outpatients seeking medical attentions.

Objectives

In our study, we investigated a population of elderly smokers or ex-smokers, with chronic respiratory symptoms, i.e. dyspnea, with a previous clinical diagnosis of COPD or HF. All patients were carefully evaluated by cardiologists and/or pulmonologists, and had a final diagnosis of COPD alone, HF alone, or concomitant COPD. The main aim of our project was to correlate the clinical presentation and the reported symptoms to the final diagnosis, possibly identifying specific clusters indicative of respiratory or cardiac diseases, or both.

Methods

This was a two-center, observational, Italian study. Study population comprised stable elderly subjects, aged 65 or older, with a positive history of smoking (> 20pack/years), evaluated in an outpatient setting, and with a previous clinical diagnosis of either COPD or HF. Subjects unwilling and/or unable to comply with study procedure and follow-up were excluded. After enrollment, participants underwent several clinical investigations, including demographic data, symptoms evaluation, 6MWT, basic biochemistry, and assessment of various comorbidities, spirometry and echocardiography. More specifically, symptoms were evaluated systematically in all patients, assessing the degree of dyspnea, cough, sputum, chest tightness, breathlessness, limitation doing activities at home, confidence in leaving home, sleep and overall energy. To provide a uniform description for all patients, the same tools were used for all participants, i.e. the mMRC and the CAT questionnaire. These questionnaires have been developed to quantify symptoms in patients with COPD, but have been used previously also in patients with concomitant heart failure[7][8][9]; more specifically, the CAT is an easy, self-administered questionnaire, which evaluates 8 different aspects of patient life, grading each one on a 0-5 scale. Spirometry, echocardiography, and the 6MWT were performed according to current international guidelines[9,10]. After completing study investigations, a final diagnosis of COPD and/or HF was made for each participant, according to the validated diagnostic criteria recommended by the International Societies (i.e. ESC for HF and GOLD for COPD)[10,11]. Patients were thus classified in three groups, COPD alone, HF alone, and COPD plus concomitant HF. All participants underwent a 3-year follow up. The study conformed to the Declaration of Helsinki and was approved by the institutional ethics committees of each participating university hospital, and all participants provided signed informed consent before recruitment.

The aim of the present study was to correlate clinical presentation (i.e. symptoms) and final diagnosis, therefore data analysis focused on the correlations between questionnaire results, clinical findings and presence/absence of COPD and/or HF. Descriptive statistics, performed with SPSS, were used. Data analysis is currently ongoing, and only preliminary results are reported.

Results

Study population included a total of 240 patients. The majority of included patients were male (75.6%), mean age was 72 years (SD 5.3), and mean BMI was 28.5 (SD 4.6). Due to inclusion criteria, all patients were either current smokers or ex-smokers (24% and 76%, respectively), with mean 50.9 ± 24 pack/years. . Not surprisingly, all patients had at least one comorbidity, and mean CCI was 5.5 (SD 1.7). For example, 18.9% of all included patients reported ischemic heart disease, 11.8% diabetes, 55.1% metabolic syndrome, and 24.4% PAD. The final diagnosis was COPD alone in 127 subjects (52.9%), HF alone in 64 (26.7%) and COPD and HF in 49 (20.4%). Mean age and BMI were similar among the three groups, while prevalence of female subjects, pack/years and CCI were significantly different among groups.

Enrolled subjects were on average only mildly symptomatic, with mean mMRC dyspnea score of 1.2, corresponding to shortness of breath when hurrying on the level and/or walking up a slight hill.

However, the first statistical analysis revealed that symptoms were significantly different among the three groups: for example, mean CAT score was significantly different among groups (p=0.001), and was the highest in patients with COPD only (13.05 ± 5.9) and the lowest in HF (9.41 ± 6.3), reaching

statistical significance at univariate analysis (p< 0.005). Surprisingly, patients with both COPD and HF had a lower CAT score than patients with COPD only, although the difference did not reach statistical significance. Similarly, patients with COPD only reported higher prevalence of cough, phlegm, and chest tightness than either patients with COPD+HF and patients with HF only (all p<0.001 among groups). Shortness of breath, on the contrary, was significantly higher in patients with associated COPD and HF (p=0.009). Interestingly, when patients were questioned about their confidence in leaving home, or their limitations doing everyday activities, or their energy, or their sleep, no differences were reported between groups.
Further statistical analysis, including correlation of symptoms with final diagnosis, multivariate and cluster analysis, is currently ongoing.

Conclusions

Although breathlessness represent the fundamental symptom of both COPD and heart failure, a careful investigation of associated symptoms may help the clinician in the differential diagnosis of these common diseases. Furthermore, certain symptoms, such as presence of chough or phlegm, strongly correlated with the presence of COPD, may alert clinicians in actively searching for this disease in the dyspneic patient.

ABBREVIATIONS

6MWT= 6-minute walk test
BMI= body mass index
CAT = COPD assessment test
CCI= Charlson comorbidity index
COPD= Chronic obstructive pulmonary disease
ESC= European Society of Cardiology
GOLD= Global Initiative for Chronic Obstructive Lung Disease
HF= Heart failure
mMRC= modified medical research council
PAD= Peripheral artery disease
SD= standard deviation

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Dr. Leda Severi

CEM Curriculum: Medicinal and Pharmaceutical Sciences
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CoTutor: Dr. Domenico D'Arca

PROTEOMICS STUDIES FOR THE IDENTIFICATION AND VALIDATION OF CELLULAR PROFILES OF NEW DRUG CANDIDATES AND DRUGS IN THERAPY

Background

Proteomics has evolved into a very powerful bioanalytical platform for the simultaneous measurement of a large number of expressed proteins, known as proteomic profile and has become an important tool for the discovery of new biomarkers useful for clinical application. Mass spectrometry proteomics can also be applied to drug discovery for the study of the mechanism of action of new drugs and for tracking the pharmacodynamic of known drugs in model systems and patients samples.

My work is focused on two main aspects: the study of the protein modulations following drug therapy in clinical patients' samples and novel drug development. These are applied to ovarian cancer translational projects and to discovery of novel drugs against trypanosomatidic infections.

Ovarian cancer (OC) represents the fifth most common cause of death from cancer in women. The standard first-line treatment consists of platinum derivatives plus paclitaxel, that, despite a high initial response, often gives rise to drug resistance onset with still unclear mechanisms. However, there is evidence that the resistance process includes the over-expression of thymidylate synthase (TS), a key enzyme involved in folate metabolism. The known TS targeting drugs in first line therapy do not show the expected efficacy because they rapidly develop drug resistance, due to the arrest of regulation function on TS-mRNA binding, however those drugs can be useful if associated with platinum drugs to prevent drug resistance. We need to identify TS targeting drugs that do not develop hTS overexpression, thus acting with a different mechanism (LR reference).

The second area of application of our proteomic studies is within the EU 7^{th}FP project NMTrypI aimed to the identification of novel drug candidates against trypanosomatidic infections (Human African Trypanosomiasis - HAT, Chagas Diseases and Leishmaniasis). In particular Visceral Leishmaniasis is an infection caused by obligate intracellular protozoan Leishmania parasites transmitted by the bite of certain sandfly species. There are an estimated 12 million humans infected. It is currently endemic in Africa, Asia and South America, and the population at risk reaches 350 million people. One of the most significant recent advances in this area has been the identification of Miltefosine (MIL), an alkylphosphocholine originally developed as an anticancer drug. MIL is far away from the optimum: its long half-life could induce the development of clinical resistance; it has been shown that MIL is teratogenic and abortifacient and this limits its use in pregnancy. This proteomic work has the aim of tracking the proteomic profile modulation due to novel drugs candidate (NMT-H0080 and NMT-A0002 compounds) to study their mechanism of action.

Objectives

The aims of my PhD work are:

ì) to identify a pharmacodynamic proteins set tracking of 5-FU efficacy in therapy within a retrospective study on serum samples from recurrent epithelial ovarian cancer (EOC) patients treated with 5-FU as third, fourth line treatment and a similar study but performed on tissue, related to treatment with Pemetrexed is planned. (project 1)

Ìì) identification and characterization of the proteins profile associated with treatments of cancer cells models with investigational TS-targeting drugs, showing a novel mechanism of action.. (project 2)

ìii) characterization of the mechanism of action of novel Miltefosine derivatives and new antiparasitic drug candidates emerging from the drug discovery project NMTrypI. (project 3).

In particular, the objective of the third year is focused on the following activities or sub-projects: ì) the identification of a protein panel that can work as biomarkers of the pharmacodynamic activity of 5-FU and a protein profile of basal samples with the aim to identify the protein set associated with the efficacy of the drug in translational study within the clinical setting studies on ovarian cancer, using a differential proteomic approach on serum samples ii) a; differential proteomic approach on serum samples of patients with platinum sensitive epithelial ovarian cancer recurrence with the aim to evaluate the Bevacizumab efficacy on second line chemotherapy (MITO16 clinical trial MITO16-MANGO- OV2 EudraCT Nr.2012-003043- 29) iii) identification of proteins target of NMT-H0080, lead compound with antiparasitic action with differential proteomic studies and fluorescence based technics.

Methods

The samples collected for the studies are: (project 1) ten serum samples collected before and after 5-FU treatment, from patients which differently responded to the treatment and 50 serum samples to be analyzed in the Bevacizumab study (project 2) and finally whole cell lysate samples from *L. Donovani* parasites treated and not-treated with new drug candidates (NMT-H0080 and NMT-A0002 compounds) (project 3).

The three sub-projects are based to mass spectrometry platforms with bioinformatics tools for the data analysis.

Serum samples analysis. Differential proteomic approach was developed in order to identify the most significant differentially expressed proteins (DEPs) within the studied samples. Serum samples were immunodepleted from IgG and Albumin through suitable kit. The resulting samples lacking of most abundant proteins, were processed as following.

Parasitic and cancer cells analysis. Protein lysates extracted both from parasites, frozen ovarian cancer biopsies and serum samples, were digested with Filter-Aided Sample Preparation (FASP) protocol, which combines the advantages of in-gel and in-solution digestion for mass spectrometry-based proteomic studies. MS analysis were performed on High-Definition (HD) ultra-high resolution (UHR) QTOF mass spectrometer (Bruker) at University of Milan Bicocca (collaboration prof. Fulvio Magni). MS data elaboration and identification of DEPs was performed with Progenesis. The

understanding of the biological systems in which DEPs are involved is very important to understand the metabolic pathways involved in drugs' mechanism of action; R and Panther software were employed to identify the most important biological process involved in the pharmacodynamics drugs profiles.
Moreover, NMT-H0080 mechanism of action will be evaluated with MS proteomics and as gel electrophoresis in not denaturing condition, in order to isolate the protein targets bound to the compound. Databases will be porously implemented for Leishmania organism based on the existing ones.

Results

Results from project 1. Identification of a pharmacodynamic proteins set tracking pemetrexed treatment response from ovarian cancer patients. On pre-treatment biopsies samples 1200 statistically significantly modulated proteins were identified. The differentially expressed proteins were classified in 19 biological process and 10 of them are selected such as biological process mainly involved in the drug mechanism of action. After network data analysis, a panel of 24 proteins was identified. As a selection methods for MS data we applied the consistency of the protein selection process with a protein set of references preliminarly designed. Western blot evaluation of 3 validated targets for pemetrexed efficacy were found consistent with our proteomic studies and literature reports. The paper is almost ready for submission.(project 1)
Regarding sub-project ì) I started the first phase. The serum samples regarding the identification of a pharmacodynamic proteins set tracking of 5-FU efficacy in therapy within a retrospective study on serum samples from recurrent epithelial ovarian cancer (EOC) patients treated with 5-FU as third, fourth line treatment, were prepared, the protocol for the serum treatment was set-up and MS analysis were started.
Results from project 3. Miletfosine and its analogues have been characterized for their protein set modulation. The work was completed even if new round data are expected for results confirmation. I have also started sub-project regarding the identification of molecular profile of antiparasitic agents, the principal problem is proteins availability in database because many parasitic proteins are uncharacterized or their function is not classified. So, 406 proteins were identified and quantified. 17 modulated proteins were obtained and their involvement in the NMT-H0080 mechanism of action is ongoing. A new round of measurements is ongoing, so the results need to be confirmed. The fluorescence-based experiments (COST action1307 in Hamburg) on NMT-H0080 show its interaction with proteins into mitochondrial, intermediate and cytoplasmic subcellular compartments. Refined work is expected to be developed on the 3rd year.

Dr. Angela Toss

CEM Curriculum: *Translational Medicine*
Tutor: Prof. Massimo Federico

STRATEGIES TO PREDICT TREATMENT RESPONSE AND SELECT THERAPIES IN METASTATIC BREAST CANCER PATIENTS USING A NEXT GENERATION SEQUENCING MULTI-GENE PANEL

Background

Breast cancer continues to be the most common form of cancer that affects women worldwide. Despite the slight improvement in overall survival observed in the last two decades, the prognosis of patients with metastatic disease continues to be poor [1,2]. In the last few years, the standard of care for patients with metastatic breast cancer has gradually evolved from empirical treatments based on clinical-pathological characteristics to the use of targeted approaches based on the molecular profile of the tumor. Consequently, an increasing number of molecularly targeted drugs have been developed for the treatment of metastatic breast cancer. These drugs target specific molecular abnormalities, including mutated protein kinases and amplified or rearranged transcription factors [3]. These alterations, called '*driver mutations*', confer to cancer cells a survival advantage, so that targeting these alterations is a rational strategy to offer more personalized and effective treatment to metastatic patients [4]. Nevertheless, what is still missing is, first of all, a tool indicating which treatment should be proposed and administered to patients and secondly, one able to predict their response to therapies. On the other hand, recent advances in technologies are expanding the scope of personalized medicine by providing new opportunities to develop more complete and dynamic diagnoses. Particularly, next generation sequencing (NGS) represents the most promising approach among those currently available [5]. As a result, the ability to perform multi-gene testing for a range of recurrent molecular alterations provides the unique opportunity to clarify the mechanisms of treatment resistance, to find the strategies to overcome that resistance and thus, to identify patients eligible for matched targeted therapies.

Objectives

The main purpose of our study is to investigate the mechanisms of resistance to treatments in metastatic breast cancer patients and, at the same time, to develop a tool able to select the most appropriate treatment based on the tumor molecular profile. In addition, secondary aims are to define the genomic profile of good responders or patients at increased risk for relapse and, possibly, to identify new targets for drug development.

Methods

In this study, we evaluate a panel of 25 genes involved in the mechanisms of endocrine and targeted treatment resistance. We analyze formalin-fixed and paraffin-embedded (FFPE) tissues of primary hormone receptor positive and/or HER2 positive breast cancers and the matched tissues taken from relapsed sites. Therefore, we evaluate tissues at the diagnosis and at the metastatization,

after the progression to treatments, using a next-generation sequencing (NGS) technology, the Ion Torrent Personalized Genome Machine (PGM) (Life Technologies, Guilford, CT, USA).

Results

To date, we have completed the sequencing of 7 patients (14 samples): one patient with stage IA breast cancer at the diagnosis, 2 with stage IIA, 2 with stage IIB and 2 with stage IV. Four patients had Luminal A-like breast cancer while, 3 had Luminal B-like breast cancer, 2 of which were HER2 positive.

Ten different genes (*PTEN, PIK3CA, mTOR, ERBB2, ERBB3, MET, INPP4B, MAP2K1, CDK6, KRAS*) in 6 patients showed possible damaging variants. Particularly, 4 patients showed the same mutational status in primary and secondary tumor, while 3 patients changed their mutational profile with the disease progression. In 3 early stage patients, the mutations found in primary tumors (*PTEN, PIK3CA, ERBB2*) may explain the subsequent treatment resistance and thus the relapse. In 1 patient, the metastatic site presented 3 new variants (*ERBB2, ERBB3, KRAS*), which may explain the previous treatment resistance and thus the progression of the disease.

Conclusions

Overall, in 3 patients the mutational status of primary tumor could have predicted treatment resistance and thus progression, while in 4 patients the mutational status of metastatic site could have guided differently subsequent treatment choices.

The study is open and we are currently recruiting other patients. Final results and conclusion are still pending.

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Cycle XXXI

Dr. Laura Anselmi

CEM Curriculum: Translational Medicine
Tutor: Prof. Sandra Marmiroli

RELATIONSHIP BETWEEN METABOLIC AND PI3K/Akt/mTOR SIGNALING FEATURES IN WILD-TYPE OR MUTANT NOTCH/PTEN T-ALL CELLS

Background

T-Acute Lymphoblastic Leukemia (T-ALL) is a heterogeneous malignant hematological disease, characterized by the abnormal accumulation of T-cell progenitors. Despite many efforts in designing novel treatment protocols, combined with traditional chemotherapy, prognosis of T-ALL patients with chemoresistant or relapsed leukemia is still very poor. Moreover, several key signaling pathways are deregulated in T-ALL, such as the PI3K/mTOR cascade, downstream of Notch1 mutations (found in >60% patients) or PTEN gene deletion/inactivation. These alterations frequently lead to reprogramming of metabolism, whereby cancer cells display glycolytic features even in normoxic conditions to boost rapid growth and energy demand. In spite of the heterogeneity of this malignancy, however, hitherto most patients are still treated with conventional chemotherapy regimens.

Previous studies performed by our laboratory demonstrated the importance of phosphorylome analysis of acute leukemia patients for a more effective therapy (Bertacchini J. et al., Leukemia, 2014), while the efficacy of the combined targeting of cellular metabolism and PI3K/Akt/ mTOR signaling was also verified in the context of primary effusion lymphoma cells, which exhibit a highly glycolytic phenotype (Mediani L. et al., Oncotarget, 2015).

Objectives

Based on our previous experience and on the abovementioned recent findings, the broad aim of our study is to find novel therapeutic protocols for T-ALL patients, designed according to their molecular hallmarks.

Specific aims: 1) To describe the signaling and metabolic profile of both primary cells from T-ALL patients and T-ALL cell lines, with particular attention to wild type Notch1 or/and PTEN versus mutated/deleted Notch1 or/and PTEN.

2) According to individual profiles from point (1), to examine whether combining signaling inhibitors (Notch1 or PI3K/mTOR inhibitors) with glycolysis/glutaminolysis inhibitors represents an alternative therapeutic approach.

Methods

We used an *in vitro* model represented by a panel of highly characterized T-ALL cell lines, recapitulating the heterogeneity of T-ALL phenotypes, to describe their signaling and metabolic profile. By means of specific inhibitors (the glucose analog/hexokinase inhibitor 2-deoxy-D-glucose and the dual PI3K/mTOR inhibitor PF-4691502) we examined the responses to both individual and

combined treatments, through viability assays, and software Calcusyn to evaluate any synergistic effect. Specific inhibition and interesting checkpoints (as cleaved Notch1) were verified by Western Blot. We analyzed all the cell lines by reverse phase protein array (RPPA), using antibodies to key molecules of PI3K/Akt/mTOR and MAPK/ERK cascades. We also measured L-lactate secretion in the growth medium, indirect index of glycolytic rate, by a colorimetric assay. To assess cell ability to proliferate, both in presence and absence of inhibitors, we performed colony-forming unit (CFU) assays.

Results

Overall, our results, though preliminary, indicate that cells carrying both Notch1 and PTEN mutations display higher signaling and a more glycolytic phenotype, compared to those with wild type and/or a single mutation. Besides, in these cells 2-DG and PF-4691502 show strong synergistic cytotoxicity. Conversely, cells carrying mutant/cleaved Notch1 alone are more sensitive to 2-DG as monotherapy, indicating that Notch1 may be more effective in driving metabolic rewiring; on the other side, cells carrying mutant PTEN alone are sensitive to PF-4691502, indicating a prevailing role of the signaling overactivation in these cells.

Conclusions

Overall, this project will correlate mutational and phosphorylome analysis with T-ALL metabolic phenotypes, allowing to define individual profiles and to predict specific treatments effectiveness.
As in the meantime we have also collected primary cells from T-ALL patients (both adult and pediatric) at diagnosis, we will therefore validate the above results in primary blasts.

Dr. Isabella Campanini

CEM Curriculum: Health Sciences
Tutor: Dr. Annalisa Bargellini

FEASIBILITY AND PREDICTIVE PERFORMANCE OF THE HENDRICH FALL RISK MODEL II IN A REHABILITATION HOSPITAL. A PROSPECTIVE STUDY OF DIAGNOSTIC ACCURACY IN NEUROLOGICAL, ORTHOPEDIC AND RESPIRATORY REHABILITATIVE WARDS

Background

Falls are a common adverse event in hospitals and may cause permanent disability resulting in an extended length of stay, fractures, death, decline in quality of life and increased health care costs [Ohde, 2012]. The prevalence of falls in acute hospitals ranges between 2 and 6% and in general rehabilitation settings shifts up to 20-30% [Frisina, 2010; Ross, 2012]. In fact, patients participating in rehabilitation programs, may experience falls due when perusing independence and mobility, which challenges multiple systems of balance and can increase the risk of falling [Salamon, 2012]. Several fall risk assessment tools have been developed and validated for geriatric inpatients and the Hendrich Fall Risk Model II (HIIFRM) is adequate for assessing the risk of falls for geriatric inpatients, but it has not been tested in rehabilitation wards [Hendrich, 2003; Heinze, 2006; Kim, 2007]. The aim of the study is evaluating feasibility and predictive performances of the HIIFRM in the prediction of fallers and non-fallers in a rehabilitative hospital.

Methods

This is a prospective study in the Rehabilitation Hospital of Correggio (AUSL of Reggio Emilia), with Orthopedic, Pulmonary, and Neurological Rehabilitation Units. During 6 consecutive months, all patients admitted (N=191) were enrolled in this study. Upon admission, the patients' risk of falling was evaluated by means of the HIIFRM tool within 24 hours. The occurrence of falls was verified on a daily basis. HIIFRM feasibility was assessed as the percentage of successful administrations at admission.

HIIFRM predictive performances were determined in terms of: area under the ROC curve (AUC), best cutoff, sensitivity (Se), specificity (Sp), positive and negative predictive values (PPV, NPV), along with their asymptotic 95% confidence intervals (95%CI).

Results

HIIFRM feasibility at rehabilitation units was 77%. Failure was mainly due to minimally conscious and vegetative states admitted at neurologic rehabilitation wards. Out of the 147 assessed patients 11 fell (7.5%). AUC was 0.799 (95%CI 0.685-0.873).

The original cutoff of 5 led to Se=100%, Sp=49%(40-57%). The best cutoffs, based on the lower distance from the best classification point, were 7 and 8. These led to Se=82%(74-88%), Sp=66%(58-74%), PPV=16, NPV=98 and to Se=73%(65-80%), Sp=72%(64-79%), PPV=17, NPV=97, respectively. Hence, the HIIFRM with a tuned cutoff can be used to assess the fall-risk in rehabilitative settings.

The low number of subjects included in the study and the low number of falls recorded (N=11) require caution when generalizing our results, even if the fall rate was congruent to that in literature.

Conclusions

This is the first prospective study testing the HIIFRM in rehabilitation settings (in neurological, orthopedic, pulmonary rehabilitation wards). A tuned cutoff can be used to assess the fall-risk in rehabilitative settings. Consequently, based on these results, the prediction of falls among all hospital wards, with high fall occurrence, could be achieved by means of a unique tool and two different cutoffs: 8 in the rehabilitation wards and 5 in the remaining wards.

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Dr. Anna Maria Costa

CEM Curriculum: Translational Medicine
Tutor: Prof. Giuseppe Biagini

CHARACTERIZATION OF p-ERK1/2 AND PPARγ AS MARKERS OF RESPONSE TO THE ANTICONVULSANT EP-80317

Background

Epilepsy is a major neurological disorder presenting the same prevalence as type I diabetes mellitus. The most frequent type of epilepsy is the temporal lobe epilepsy (TLE), which is associated with diffuse brain injuries, especially within the hippocampus, cognitive deficits and drug refractoriness. Overall, up to 35% of adult patients affected by epilepsy are resistant to antiepileptic drugs (AEDs).

The worldwide emergence of extensively drug-resistant epilepsy has heightened the need for new and efficient AEDs. Recently, there has been growing interest on the role of ghrelin and its receptor (GHS-R1a) in the modulation of epileptic activity. It is known that the inactive form of ghrelin, desacyl-ghrelin, is able to partially prevent status epilepticus (SE) in animal models, such as the pilocarpine model. Like desacyl-ghrelin, the GHS-R1a antagonist EP-80317 has also anticonvulsant effects in animal models of SE. However, it is not clear if these effects are based on a mechanism depending on GHS-R1a modulation. Indeed, EP-80317 is not only able to bind GHS-R1a but also to activate other signalling pathways, alternative to GHS-R1a. For instance, EP-80317 was shown to activate the peroxisome proliferator-activated receptor gamma (PPARγ) through the interaction with CD36 scavenger receptors of macrophages in the arterial wall.

Objectives

Although considerable research has been devoted to establish the anticonvulsant properties of GHS-R1a ligands, rather less attention has been paid to the signalling pathways activated by these ligands in the central nervous system (CNS). Therefore, the present study aimed to elucidate the downstream molecular mechanisms through which EP-80317 could promote its anticonvulsant effects. In particular, we have characterized the expression of PPARγ in the hippocampus in seizure animal models. Moreover, we analyzed the expression of phosphorylated extracellular signal-regulated kinase 1/2 (p-ERK1/2), a marker of neuronal activation.

Methods

Firstly, we studied the effects of pharmacological modulation of EP-80317 in the pilocarpine model of SE. Secondly, by using the repeated 6-Hz corneal stimulation model we demonstrated that the anticonvulsant effect of EP-80317 is found in different models. In both the analysed models: i) the pharmacological properties of EP-80317 were assessed by behavioral and video electrocorticographic (ECoG) analyses; ii) PPARγ involvement in EP-80317 effects was characterized by using a PPARγ antagonist (GW9662). Finally, we analyzed the immunohistochemical expression of PPARγ and p-ERK1/2 in specific hippocampal regions of mice exposed to repeated sessions of 6 Hz corneal stimulation.

Results

In the pilocarpine and the repeated 6-Hz corneal stimulation model, administration of EP-80317 was able to antagonize seizures. Pre-treatment with GW9662 prevented most of EP-80317 anticonvulsant effects. Additionally, it decreased the latency to develop stage 4-5 seizures and accelerated the development of SE in the pilocarpine model. In the hippocampus of mice exposed to repeated sessions of 6-Hz corneal stimulation, the anticonvulsant effect of EP-80317 was associated with the prevention of the increase in levels of p-ERK1/2 observed in control mice and, additionally, with increased levels of PPARγ. In particular, the observed effects on p-ERK1/2 and PPARγ immunoreactivity were directly related to the pharmacological activity of EP-80317. However, these effects of EP-80317 vanished by repeating the sessions of seizure induction up to four. The transient effect of EP-80317 on the overall seizures suggested the appearance of refractoriness to the tested anticonvulsant.

Conclusions

These results suggest that EP-80317 has anticonvulsant effects which involved modulation of p-ERK1/2 and PPARγ expression. Moreover, these anticonvulsant effects seem to decrease in presence of recurring seizures, a phenomenon associated with normalization of PPARγ expression and increase in p-ERK1/2 expression.

Dr. Francesca Faillaci

CEM Curriculum: Translational Medicine
Tutor: Prof. Erica Villa

HEPATOCELLULAR CARCINOMA IN A ZEBRAFISH MODEL: EXPLORATION OF THE ROLE OF INFLAMMATION, NEO-ANGIOGENESIS AND BIOLOGICAL AGGRESSIVENESS

Background

Hepatocellular carcinoma (HCC) is the most frequent primary malignancy of the liver, totaling to more than 95% primary liver tumors in the adult. HCCs are phenotypically and genetically heterogeneous tumors that commonly emerge on a background of chronic liver disease (CLD) and cirrhosis. Patients with aggressive HCC can be identified by means of neo-angiogenic 5-genes transcriptomic signature (1), which is strongly related with rapid progression of tumor, risk of recurrence after therapy, and extremely low median survival.

Looking for suitable experimental models, which recapitulate human events, we selected Zebrafish (*Danio rerio*) as an experimental model in the attempt to create conditions similar to human carcinogenesis. As fatty liver disease is becoming the leading cause of chronic liver disease (CLD), we first set up an overfeeding model in Zebrafish to evaluate the pure effect of excess caloric intake. We demonstrated that overfed fish (obese) develop steatosis and, despite lack of inflammation, fibrosis but not liver cancer (2).

Objectives

1. To investigate the effect of the addition of an inflammatory stimulus (TAA) to the development of liver cancer in overfed Zebrafish but not in lean ones
2. To study the HCC microenvironment in the determination of tumor aggressiveness both in the experimental model and in humans
3. To evaluate the clinical outcome in humans according to microenvironment's features

Methods

1. To investigate the role of inflammation in overfed Zebrafish we used as inflammatory stimulus the hepatotoxin thioacetamide (TAA) (3). Young male and female overfed Zebrafish were injected with 300mg/kg of TAA for 12 weeks. As control we used TAA injected fish under standard diet (2). Histopathological examination and Real Time RT-qPCR on genes involved in inflammation (IL-6, TNFα) and HCC (c-MYC, Gankyrin (also known as PMSD10), IGF-2, AKT, mTOR, AMPKa) were performed on liver samples collected at 1, 6, 8, 12 weeks. Hepatic E-cadherin expression was studied by immunohistochemistry.
2. To further biologically characterize the microenvironment of human HCC, in order to have additional tools to better define its biological aggressiveness and the correlation between mRNA, protein abundance and co-expression of micro-environmental factor,

immunohistochemistry (IHC) on paraffin-embedded liver section (HCC and surrounding tissue) and western blot on protein lysates from frozen samples were carried out. In detail ANGPT2, PD-1, PDL-1, CLEC-2 and E-Cadherin antibodies were used for Immunohistochemistry and for western blot analysis.

Results

1. Experimental Zebrafish overfeeding/TAA model: TAA-injected fish developed increased hepatic inflammation, up-regulation of cytokines IL-6/TNFα and bile duct dilation from week 6 of treatment. Fibrosis and increased intrahepatic collagen occurred only in males at week 6. Overfed/TAA males displayed strong hepatic hyperplasia of liver tissue at time point of week 8 and multifocal HCC at week 12. E-cadherin staining was progressively lost in males treated fish.
2. Human HCC: A marked local up-regulation of both PD1 and PDL-1, a loss of E-cadherin, gain of epithelial-mesenchymal transition (EMT) phenotype, and extreme poor differentiation at histology were present. Transcriptomic analysis evidenced a significant down-regulation of CLEC2. Interestingly, this data is related with presence, at western blot, of low molecular weight form of the protein, corresponding to deglycosylated one. Up-regulated microRNA in fast growing HCCs are associated with TGF-β signaling, angiogenesis, and inflammation.

Conclusions

The model that we have set up develops liver cancer in a matter of 12 weeks, and this is likely in relationship with the high level of inflammation determined by the TAA protocol, which we used. Development of low-level inflammation model in zebrafish is in progress. We also plan to use inhibitors of analyzed pathways to verify a) whether it is possible to slow down liver damage progression, b) identify similarities between human and zebrafish HCC.

Our data show that fast HCCs are characterized not only by redundant neo-angiogenesis but also by features of immunosuppressed microenvironment, epithelial-mesenchymal transition (EMT), and TGF-beta pathway activation in a permanent inflammatory state.

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Dr. Francesco Fontana

CEM Curriculum: Translational Medicine
Tutor: Prof. Gianni Cappelli

MONOCLONAL GAMMOPATHY OF UNDETERMINED SIGNIFICANCE AFTER KIDNEY TRANSPLANTATION: SINGLE CENTER EXPERIENCE

Background

Monoclonal gammopathy of undetermined significance (MGUS) is an asymptomatic pre-malignant plasma cell disorder defined by the presence of a monoclonal immunoglobulin in serum, and is associated with an increased relative risk for later development of multiple myeloma (MM) and other lymphoproliferative diseases. Prevalence of MGUS is reported to be about 3-4% in the general population above 50 years of age. Although MGUS has been largely evaluated in general population-based studies, its prevalence and course in organ transplant recipients remains undefined. Prevalence, associated factors, clinical characteristics and prognosis of MGUS after kidney transplantation (KT) have been assessed in few studies, with conflicting results.

Objectives

Our aim was to assess the prevalence of MGUS in a population of KT recipients, evaluating its association with clinically significant outcomes.

Methods

We retrospectively reviewed medical charts of 548 patients who received a KT between January 1998 and December 2015 at “Policlinico” hospital in Modena, Italy. Diagnosis of MGUS was suspected on the basis of a positive serum protein electrophoresis, and subsequently confirmed and typed by immunofixation. We analyzed demographics and monoclonal component characteristics of KT recipients diagnosed with stable MGUS after KT, comparing this group of patients with a control population of 79 KT recipients without MGUS matched for sex, age and transplant date in a matched case-control analysis.

Results

Thirty-nine patients (8.1% of analyzed population) developed a stable MGUS after KT. The mean age at diagnosis was 57 ± 9.7 years, and 23% of patients were younger than 50 years. We found no sex difference in the prevalence of MGUS after KT. The median time from KT to the appearance of MGUS

was 5.3 ± 4.6 years during a median follow-up of 5.9 ± 5.2 years. Monoclonal protein was most often of the IgG subtype (54%); of note, it was bi-clonal in 26% of the 39 patients, and in one patient (2%) was tri-clonal. The mean serum monoclonal component concentration was 0.58 gr/dl, with values ranging from immeasurable to 2.1 gr/dl; serum levels of monoclonal proteins remained substantially stable over time. MGUS progressed to fatal MM in one patient, no patient had bone marrow biopsy or laboratory characteristics suggestive for smoldering myeloma and there was one case of PTLD. In the matched case-control analysis the incidence of hematological and solid neoplasms was similar in both groups; on the other hand, MUGS patients had a significantly higher prevalence of Monoclonal B-cell lymphocytosis (MBL), an asymptomatic premalignant condition characterized by the expansion of a clonal B-cell population. The rate of serious infectious events was slightly higher in the MGUS group, although the difference was not statistically significant. There was no difference in the overall incidence of biopsy-proven rejection; it is worth to note that ten cases of rejection (91%) preceded the diagnosis of MGUS by a mean age of 5 years. KT patients with MGUS had a slightly higher risk of developing CKD stage IV, but the difference with the control group was not significant. Graft and overall survival after KT were not significantly different among patients with MGUS and controls. In the univariate logistic regression, the only factor potentially associated with the development of MGUS after KT was a positive history for inflammatory kidney disease before KT.

Conclusions

We report a high incidence of stable MGUS after KT (8.1%). Differing from general population, the occurrence of MGUS was not influenced by age and gender, with a considerable number of cases developing in young subjects. The presence of MGUS did not impact on graft survival, overall survival and incidence of malignancies. Although MGUS did not show a very aggressive course in terms of frank progression to MM, we found a significant increase in the incidence of MBL, pre-malignant condition which warrants a strict hematological follow-up in KT patients. The only predictor for the development of MGUS was a pre-transplant history of inflammatory kidney disease; this finding, together with the evidence of frequent episode of steroid-treated rejection preceding the diagnosis of MGUS, suggests the hypothesis that a higher burden of cumulative immunosuppression would possibly play a role in the development of the monoclonal gammopathy.

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NEW INSIGHTS INTO SKIN PHYSIOLOGY

Background

Skin physiology includes the regulation of several activities. Among these, pigmentation, proliferation, vascular regulation and immunity. A disruption of regulatory mechanisms can occurr, with passing years, leading to the development of different types of skin changes.

Objectives

The main purpose of my research is to explore the effect of some gene mutations in skin physiology, focusing on skin aging and skin cancers. Non-invasive skin imaging correlations can also be used in order to provide new insights into morphological variations of both epidermis and dermis.

Methods

100 women were enrolled in our study, after achieving informed consent. An analysis of genes involved in metabolism, vascular regulation, oxidative stress and inflammation was performed. Reflectance confocal microscopy (RCM) and optical coherence tomography (OCT) images were also collected.

Results

Preliminary results show the significant correlation between some mutations and specific patterns revealed by means of non-invasive skin imaging techniques. Further results will be provided by the complete analysis of our data.

Conclusions

Morphological skin variations and genetic data can be correlated with individual characteristics of subjects enrolled in the study. These correlations have not been previously explored and will be used in the construction of a model of aging in subjects with different phototypes, providing an overview of progressive physiological and pathological changes of the skin.

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PALLIATIVE CARE FOR HOSPITALIZED GERIATRIC PATIENTS:

A NEW EXPERIENCE IN THE GERIATRIC PALLIATIVE UNIT OF MODENA HOSPITAL

Background

Palliative care (PC) is an approach that improves the quality of life of patients and their families facing the problem associated with life-threatening illness, through the prevention and relief of suffering by means of early identification, assessment and treatment of pain and other problems, physical, psychosocial and spiritual. PC has recently spread in geriatric settings because of the increasing number of elderly patients affected by end-stage chronic disorders addressing to hospitals at the end of life. To offer high quality PC, a new Geriatric Palliative Unit (GPU) raised in Modena Hospital (Ospedale Civile S. agostino-Estense), consisting of 3 hospital beds. Here a multidisciplinary team (geriatrician, nurse, healthcare assistant, physiotherapist, psychologist, ethicist) takes care of end-stage patients and their families, with a palliative approach.

Objectives

The principal aim of the study is to demonstrate the higher quality of PC performed in a dedicated setting (GPU in Modena Hospital) compared with hospital standard treatment.

Methods

We carried out a perspective study enrolling 69 end-stage patients treated in the GPU during the first 5 months of its activity (October 2015-February 2016). For each terminal patient the decision of starting a palliative treatment was shared between the medical team and patient's relatives. Patients came to the GPU from the Emergency Department, a Medical Ward or an Intensive Care Unit. In the GPU a multidisciplinary team offered PC aimed to symptoms control and patient's relatives support, in a customizable setting. The Edmonton Symptom Assessment Scale (ESAS) was used for symptoms monitoring, and the Zarit Scale to screen relatives' stress. Comorbidities and functional status were recorded for each patient. Moreover, traditional care in the ward of provenance was compared with PC in the GPU.

Results

The 69 patients admitted to the GPU aged 83±8 years (58% were females). They showed a high comorbidity index and a low functional status (Charlson Index: 5±2, Activities of Daily Living: 1±2). The more frequent end-stage conditions were: dementia (32%), cerebral stroke (32%), tumor (13%) and chronic heart failure (10%). 68% of the patients came to the GPU from a Medical Ward, 20% from the Emergency Department, and 12% from an Intensive Care Unit. The mean length of GPU stay was 5,2±4,7 days, corresponding to 52±32% of total hospital stay length. 3% of the patients was discharged and died at home. Compared with traditional care performed in the ward of provenance, PC offered in the GPU showed a statistically significant reduction of diagnostic tests prescription, artificial nutrition and drugs administration, and a statistically significant increase of pain medications/antiemetics/hypnotics use. Symptoms monitoring through the ESAS revealed a significant better pain and dyspnoea control. Symptoms were controlled using both a pharmacological and a not-pharmacological (physiotherapist's and psychologist's contributions) approach. The psychologist's role was crucial for the support offered to patients, relatives, and the medical team. The psychological support resulted in a reduced rate of depressive reactions among patients' relatives.

Conclusions

The relevant number of patients admitted to the GPU demonstrates the growing sensitivity of hospital physicians toward PC. The right of receiving PC for patients with end-stage conditions is spreading among healthcare professionals. PC offered in the GPU by a multidisciplinary team consists of limited diagnostic/therapeutic performances, better symptoms control through both medical and assistive approaches, and stronger relatives' support. Considering the interesting preliminary results of the GPU experience, further resources should be invested to answer the increasing demand of hospital PC and to improve healthcare professionals' knowledge of PC.

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EFFECTIVENESS OF MONOCHLORAMINE IN REDUCING *LEGIONELLA* WATER CONTAMINATION AVOIDING FORMATION OF BY-PRODUCTS

Background

The control of *Legionella* spp contamination in plumbing systems of large buildings may be a challenge. This is particularly relevant in hospital settings where the contamination of hot water distribution systems is the primary risk factor for nosocomial *Legionella* pneumonia[1,2]. Several technologies have been used to disinfect hospital water systems contaminated by *Legionella*[3]. Among the others, monochloramine has been recently applied in hospital plumbing systems by our study group with satisfactory results[4].
Studies conducted in vitro and/or on municipal water distribution system reported the presence of nitrogen by-products in chloraminated waters[5,6]. Particular attention has been devoted to evaluate production of nitrosamines for their toxicological consequences on human health[6].

Objectives

This study evaluates the effectiveness of three monochloramine devices, which differ in length of activity and injection method of the biocide, in reducing *Legionella* spp water contamination, measuring the possible modification of water physicochemical parameters and the formation of disinfection by-products.

Methods

Three devices applied in the same hospital building were investigated: device A has been working since 2009, B and C were installed for this study in water networks previously treated with chlorine dioxide. Monochloramine is either prepared in situ in hot water (A and B) or prepared and injected in the cold make-up water (C). Municipal cold water and hot water from return-loops of treated and untreated pipeworks were collected. *Legionella* spp was quantified by culture method (ISO 11731:1998). *Legionella* monitoring was performed concurrently with physicochemical monitoring by standard methods. Physicochemical parameters included temperature, pH, monochloramine, free-ammonia, TOC, bromide, nitrite, nitrate, N-nitrosamines, trihalomethanes, chlorite and chlorate.

Results

During 18 months of the study, *Legionella* was occasionally found at low levels in hot waters. Nitrates below 50 mg/l were measured in all samples, including those treated with monochloramine. Nitrosamines were detected only in the network A, treated for a long time with monochloramine,

but residuals did not exceed the limit (0.10 µg/l). Levels of chlorate >700 µg/l and ammonia > 0,50 mg/l were observed in pipeworks treated by monochloramine, but parameters were decreased below limit value after corrective actions implementation. Corrective actions included draining and cleaning of storage tanks and replacement of chlorine reagent with fresh product. No nitrite, bromide and TOC were detected, and THMs were extremely low.

Conclusions

Our results confirm the efficacy of monochloramine in controlling *Legionella* contamination. During 18 months of study, no formation or increase of undesirable DBPs such as chlorite, N-nitrosamines and THMs or precursor compounds such as nitrites and nitrates was observed. High levels of chlorate and ammonia have been measured suggesting an incomplete reaction of the precursor compounds of monochloramine, due to high temperatures in the storage area. As well as for all disinfection systems, we highlight the importance of system monitoring such as the control of reagents storage (temperature and tanks) in order to ensure correct dosing proportions.

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HIV-INFECTED PATIENTS WITH LOW CD4/CD8 RATIO SHOW ALTERED EXPRESSION OF THE INFLAMMASOME COMPONENTS AIM2, PYCARD AND IL-1B

Background

Persistent and chronic level of inflammation is a hallmark of the HIV infection. Indeed, even a successful and effective combination antiretroviral therapy (cART) is unable to avoid a chronic inflammatory status that persists at a low grade. Inflammasomes and innate mitochondrial sensing pathway could be crucial for triggering and maintaining inflammation. In particular, PYCARD is the mediator of assembly of inflammasomes. AIM2 and NLRP3 are principal sensors for damage, not only because of infections, but also due to metabolic stress and tissue damage. The first senses the double-stranded DNA in the cytosol, while the second leads to the activation of pro-caspase 1 and, finally, to the secretion of two inflammatory cytokines, IL-1 β and IL-18. Monocytes are key players in innate immunity, including inflammatory phenomena, and intervene in the regulation of adaptive immunity.

Objectives

To clarify, at least in part, the role of inflammasomes in immune reconstitution, by studying mRNA expression and cytokine secretion levels of the main components of inflammasomes in monocytes from successfully treated HIV+ patients with undetectable viremia but different CD4/CD8 ratio.

Methods

We enrolled 26 patients, 11 with a low CD4/CD8 ratio (<0.4) and 15 with a high ratio (>1.2). Highly purified monocytes were stimulated with LPS for 1 and 4 hours. Total RNA was extracted and reverse transcribed for the analysis of AIM2, NLRP3, NAIP, PYCARD, IL-18, IL-1β besides 3 reference genes (ACTB, TBP and RPS18). IL-1β and IL-18 were quantified by ELISA on monocyte culture supernatants from 5 patients for each group.

Results

AIM2 levels increased in both groups after 4h of stimulation, more strongly in patients with high CD4/CD8 ratio. In this group, IL-1β mRNA levels also increased more than in patients with low ratio. NLRP3 levels increased similarly in both groups, after 1h of stimulation. PYCARD levels decreased in 4h-stimulated cells of patients with high ratio. Supernatant levels of IL-1β increased in both groups after 4h LPS stimulation, while IL-18 remained stable.

Conclusions

HIV-Infected patients with low CD4/CD8 ratio showed altered expression of the inflammasome components AIM2, PYCARD and IL-1β. This could indicate a diminished capability to recognize nucleic acids. The presence of similar levels of cytokines may be due to the low number of patients analyzed. Optimal activation of inflammasomes in monocytes could play a crucial role in the immune reconstitution. This may provide insights into the development of potential therapeutic targets. We are at present performing western blot analysis to investigate possible changes even in protein expression, particularly in AIM2, NLRP3 and PYCARD.

Dr. Cinzia Puzzolante

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ROLE OF PET-CT IN EARLY INTERIM ANALYSIS OF PATIENTS WITH NATIVE VERTEBRAL OSTEOMYELITIS

Background

Native vertebral osteomyelitis (NVO) is a rare but serious disease with rates of in-hospital mortality and long-term sequelae of 6 % and 7% respectively. According to IDSA guidelines, released in 2015, a six-week course of ABT is recommended for most patients with bacterial NVO; tubercular (TB) NVO is usually treated with a 9- to 12-months course of antimicrobial therapy (ABT). No reliable tools are available for predicting ABT failure in early stages. Our aim was to determine if Positron emission tomography combined with computed tomography (PET-CT) scan performed at early stages could predict the efficacy of ABT in patients with NVO.

Methods

We performed a retrospective, observational study collecting demographic, clinical, microbiological and radiological data of patients with NVO followed at Modena Policlinico between 2010-2016. PET-CT scan at baseline had to be performed before ABT and before bone biopsy. Follow-up PET-CT scan had to be performed within 14-21 days and after 30 days for bacterial and TB NVO respectively. Standardized Uptake Value (SUV) at osteomyelitis sites was collected and compared for each patient during the follow-up. Improvement of PET-CT was defined as maximum SUV decrease ≥25% after 15 days of ABT (SUV15) from baseline (SUV0) in patients with bacterial NVO. Clinical failure was defined as necessity of ABT ≥12 weeks (until 2015) or 6 weeks (from 2016) in patients with bacterial NVO or ≥12 months in patients with tubercular (TB) NVO. Correlation between clinical improvement and PET improvement was evaluated using univariable logistic regression analysis.

Results

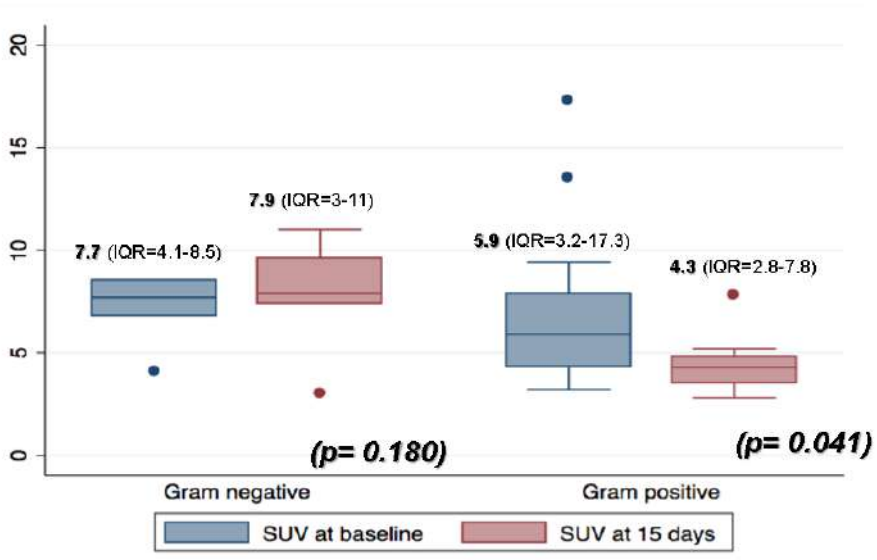
Ninety-four cases of NVO were collected. Etiological diagnosis was obtained in 73 cases (77.7%) and all were monomicrobial. TB NVO represented of 15% of cases. In 13 out of 17 patients of foreign origin *M.tuberculosis* was the etiological agent of NVO (76.5%); 22.3% had immunosuppressive co-morbidities; in 38.3% of patients lumbar site was involved. Patients with TB NVO were younger (39.5 years, IQR=28-46) and of foreign origin (92.9%) compared with patients with bacterial NVO ($p<0.001$) and had more frequently concomitant abscess (71.4%, $p=0.002$). After biopsy ABT was empirically started with broad-spectrum penicillins + rifampin or with fluoroquinolone + rifampin unless TB etiology was suspected. Mean duration of ABT was 12 weeks (IQR=8-14 weeks) for patients with

bacterial NVO, and 12 months (IQR=12-17 months) for patients with TB NVO. Forty-six patients (49%) with NVO had a baseline PET performed. Mean SUV0 value was 7 (SD=±3.6) with no difference between TB and non-TB NVO (p=0.948). In 15 patients with Gram positive-NVO median SUV0 and SUV15 was 5.9 (IQR=3.2–17.3) and 4.3 (IQR=2.8 –7.8) respectively (p=0.041); in 7 pts with Gram negative-NVO median SUV0 and SUV15 was 7.7 (IQR= 4.1-8.5) and 7.9 (IQR= 3-11) respectively (p=0.180) (see fig.1). One patient out of 14 with TB NVO underwent PET scan after 30 days from baseline and no statistical analysis was possible. No statistically significant correlation between SUV15 decline and clinical cure was found both in patients with microbiological diagnosis (p_0=0.388) and in the sub-set of patients without etiological diagnosis (p_0=1.000).

Conclusions

A decrease in SUV15 value from baseline of at least 25% does not significantly correlate with clinical cure, both in patients with clinical isolate and in patients with unknown etiology. PET-CT should not be considered a predictive clinical tool for an early interim analysis of clinical outcome of patients with bacterial NVO. The significant most rapid SUV decline in Gram-positive NVO compared to Gram-negative NVO should be further evaluated.

Fig.1



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EARLY PREDICTORS OF GRAFT SURVIVAL AFTER SIMULTANEOUS KIDNEY-PANCREAS TRANSPLANT

Background

Pancreas transplantation (PT) has become the treatment of choice for many patients with severe complications of diabetes, particularly those with end-stage renal failure. Since 1966, when the first successful pancreas transplant in humans was performed, the results of pancreatic transplantation have dramatically improved. However, graft failure rates are almost 10% at 1 year and 30% at 5 years, and complications such as pancreatitis can be life threatening. Pancreatic graft failure is strongly associated with post-transplant mortality. Effective early markers of graft dysfunction that could allow an early identification and possible treatment of pancreatic impairment are lacking.

Objectives

This study aims to identify early predictors of pancreatic graft survival and to examine DGF patterns and how do they relate to pancreatic graft survival.

Methods

This is a retrospective analysis. Data are retrieved from the intensive therapy unit (ITU) and transplant surgery databases for all whole organ pancreas transplants performed between January 2009 and December 2012 (data collection is still ongoing).

We analysed recipients demographics and donor/graft characteristics and monitored every 6 hours for the first 2 days after surgery the values of: blood glucose (BG), base excess (BE), lactate, pH, blood pressure (BP) and intravenous infusions and fluid balances.

Data were checked for missing entries; cases were excluded if pancreas graft outcomes were unknown. Graft failure was defined by a return to exogenous insulin therapy or explant of organ.

Pancreas transplantation was performed according to a standardized clinical protocol with systemic venous drainage and enteric exocrine drainage. Donors and recipients were matched according to national organ allocation guidelines. All recipients followed a standard immunosuppression protocol comprising basiliximab induction and tacrolimus, mycophenolate and steroids maintenance.

Results

Data for 80 simultaneous kidney-pancreas transplants (SPK) were retrieved and included in the analysis so far. Twenty-one transplanted grafts (26.2%) were from donors after cardiac death (DCD), with an average cold ischemia time of 13±3.2 hours, an average donor age of 35±13.2 years and an average pancreas donor risk index (PDRI) of 1.59±0.57. The recipients were 42±9 years old at the time of the transplant and with a BMI of 24.7±3.9. We observed 8 graft failures (10%) after a median time of 13.5 months (range 0-41). At the multivariate analysis of risk factors the only variables independently associated with a poor outcome were postoperative blood transfusion (HR 25.5, 95% CI: 2.07-314.5, p=0.023) and noradrenaline requirement (HR 20.3, 95%CI: 1.51-273.2, p=0.011).

Conclusions

The management of pancreas transplantation is complicated by the absence of a marker that enables graft dysfunction to be detected at an early enough stage to allow more intensive investigation or effective intervention. Blood transfusion, and noradrenaline requirement, were the only variables independently associated with pancreatic graft loss. This result may reflect the paramount importance of an adequate graft perfusion in the early postoperative period. Blood glucose levels and the other recipient and donor characteristics analysed, do not appear to have a significant influence on graft survival.

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CARDIO-PULMONARY EVALUATION IN A LARGE COHORT OF PATIENTS WITH SYSTEMIC SCLEROSIS: A RIGHT AND LEFT HEART DISEASE

Background

Systemic sclerosis (SSc) is a chronic connective tissue disease characterize by widespread microvascular damage, dysregulation of fibroblasts with collagen overproduction and excessive fibrosis of the skin and internal organs, as well as complex immune system abnormalities.

Cardiopulmonary involvement is common in SSc: pulmonary fibrosis, pulmonary arterial hypertension (PAH), electrical disorders are the most serious complications and frequent cause of death.

The heart is one of the major organs involved in scleroderma: once cardiac involvement is clinically evident, it is recognized as a poor prognostic factor and despite the availability of innovative target therapy it is one of the leading causes of mortality in SSc patients.

Objectives

To assess the type and severity of cardiopulmonary involvement and its correlations with SSc clinical features, quality of life, and survival. In particular we focused on PAH and right heart disease in scleroderma patients. Furthermore we aimed to detect possible parallels between SSc and diabetes mellitus (DM) and their impact on left heart disease because of the similar microvascular abnormalities.

Methods

We previously analysed 241 consecutive SSc patients referred to our Rheumatology Unit from January 1999 to January 2014. We recently expanded our series to 343 patients evaluated from January 1999 to December 2016 (F/M 302/41). A group of diabetic patients with similar demographic features and clinical characteristics was also enrolled. All patients underwent general and cardio-pulmonary evaluation, including demographic and clinic-serological features, standard electrocardiogram (ECG), Doppler echocardiography, right heart catheterization (RHC) when requested, high resolution scan of the lungs (HRCT), and pulmonary function tests, according to current methodologies. Traditional and non-traditional cardiovascular risk factors were also studied.

Results

Our previous results on 241 patients showed that patients of our cohort died (38) because of pulmonary complications (15.8%), severe PAH (39.5%), in 5 cases complicated by lung cancer (13.2%), cardiac involvement (26.3%), and other causes (18.4%). Statistically significant findings were found in the deceased group if compared to remaining patients. In particular, forced vital capacity (FVC) and carbon monoxide diffusing capacity (DLCO) were significantly reduced in the deceased individuals ($p<0.001$) which also showed higher rates of severe fibrosis at HRCT ($p<0.001$). Furthermore, these patients were characterized by higher incidence of ECG frequency and rhythm alterations ($p<0.001$), ischemic ECG changes ($p=0.003$), increased systolic PA-pressure, decreased values of tricuspid annular plane systolic excursion (TAPSE), reduced left ventricle ejection fraction, right-sided dilatation ($p<0.001$), higher prevalence of pericardial effusion ($p=0.012$) at echocardiography, and reduced cardiac index ($p=0.007$) and cardiac output (0.036) at right heart catheterization (RHC). After RHC, 24 patients were diagnosed with PAH. Strong correlations were found between RHC and anticentromere antibody-ACA, a marker of the disease.

Conclusions

Our previous findings confirm literature data about the prognostic value of cardiopulmonary involvement that is the leading cause of SSc-related morbidity and mortality. Further results about right and left heart dysfunctions are still pending.

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STUDY ON PROTECTIVE ACTION OF HYDROGEN SULFIDE IN RAT AND MOUSE MODELS OF ALZHEIMER'S DISEASE

Background

Alzheimer's disease (AD) is a chronic disorder characterized by progressive neurodegeneration associated with cognitive decline and several behavioral deficits. Sporadic AD is generally diagnosed in people over 65 years of age, whereas familial or genetic AD (the less prevalent form of Alzheimer) is an early-onset autosomal dominant disease [1].

In some regions of AD brains, such as cortex and hippocampus, two members of the γ-secretase complex, presenilin-1 (PS1) and presenilin-2 (PS2), and β-secretases process the amyloid precursor protein (APP) to generate extra-cellular β-amyloid (Aβ) fibrillar deposits (Aβ plaques); also intraneuronal tau neurofibrillary tangles composed of hyperphosphorylated tau protein develop. Both these hallmark lesions trigger pathophysiological pathways that lead to synaptic dysfunction, neurodegeneration and marked neuronal loss with consequent impairment in cognitive functions. Furthermore, free radicals, nitric oxide, glutamate, several cytokines, mitogen-activated protein kinases, Bcl-2 family members and caspases play an important role in the above mentioned pathophysiological pathways. A combination of impaired cholinergic transmission and high glutamate activity underlies the main symptomatology of AD, which is characterized by memory loss and severe cognitive decline [2].

Hydrogen sulfide (H_2S) is a colorless, flammable, water-soluble gas and Tabiano's spa-waters are particularly rich in H_2S (strong sulfydrometric degree, that is, more than 100 mg/l) [3]. H_2S is increasingly being considered as an important signaling molecule in various body systems, and accumulating evidence demonstrates that H_2S donor compounds exert significant beneficial effects in several animal models of inflammation and ischemia/reperfusion injury [4]. H_2S is endogenously produced also in the brain, probably exerting a neuromodulatory role. It has been previously reported in literature that brain H_2S synthesis is severely decreased in AD patients, and plasma H_2S levels are negatively correlated with the severity of AD [5]. Recent data showed that the H_2S donor sodium hydrosulfide reduces Aβ generation in cultured cells, and Aβ-induced cognitive impairment in rats, as detected in a short-term study [6]. Further, inhaled H_2S has resulted to be able to prevent neurodegeneration in a mouse model of Parkinson's disease [7].

Objectives

The first aim of my research project has been to evaluate the possible neuroprotection on cognitive effects of a short- and long-term treatment with a H_2S donor and Tabiano's spa-water to counteract

the progression of AD. To this end, in two different animal models of AD, I investigated learning and memory ability.

Methods

For my study I used two different animal models of AD:

- rat model of AD induced by an unique brain injection of β-amyloid1-40 (Aβ);
- AD mouse model harboring human transgenes APPswe, PS1M146V, tauP301L (3xTg-AD mice).

In the rat model I studied an early phase of AD, in transgenic mice I evaluated the middle AD conditions.

Animals were divided into 4 experimental groups:

- *Experimental group A* (Aβ rats): dose-response study with a donor of H_2S, 0.25, 0.5, 1 mg/Kg or saline, i.p., once daily for 15 days starting 3 hours after Aβ injection, and sacrificed at day 15;
- *Experimental group B* (Aβ rats): dose-response study with spa-water, 3, 6 and 12 ml/Kg or saline, i.p., administered for 15 days starting 3 hours after Aβ injection, and sacrificed at day 15;
- *Experimental group C* (3xTg-AD mice): spa-water, 12 ml/Kg (best dose found in rat model) or saline, i.p., administered once daily for 12 weeks starting at 12 weeks of age and sacrificed at 24 weeks;
- *Experimental group D:* control animals (rats without Aβ injection and wild-type mice received an equal volume of saline by the same route of administration).

In each model I investigated the neuroprotective effects of a H_2S donor or Tabiano's spa-water through analysis of cognitive tests (Morris water-maze test). These tests measure animal's ability to learn, remember and go to a place (platform) in space defined only by its position relative to distal extramaze cues.

Results

In Aβ rats treated with the H_2S donor (0.25, 0.5, 1 mg/Kg i.p.) or Tabiano's spa-water (3, 6 and 12 ml/Kg i.p.) there was a dose-related improvement in learning and memory performance, compared with the respective saline treated animals. The maximally effective doses of H_2S donor and spa-water were 0.5 mg/Kg and 12 ml/Kg, respectively.

Improvement in cognitive performance was also found in all four sections of the Morris test carried out in 3xTg-AD mice (only treated with 12 ml/Kg spa-water, because in AD rats it appeared more effective than sodium hydrosulfide).

Saline-treated AD control animals (Aβ rats and 3xTg-AD mice) showed impaired ability, as compared with normal controls, in platform finding during the first and second sessions (assay of learning and memory, respectively). Learning and memory impairment persisted during the third and fourth sessions (performed only in 3xTg-AD mice).

Conclusions

These favorable results would suggest that appropriate treatments with H_2S donors or Tabiano's spa-water might represent an innovative approach to slow down AD progression in humans.

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Cycle XXXII

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ASSOCIATION BETWEEN MELANOMA CYTO-ARCHITECTURAL FEATURES AND THE INTRATUMORAL MICRO-VASCULARIZATION AS DETECTED IN VIVO BY MEANS OF REFLECTANCE CONFOCAL MICROSCOPY AND SPECKLED-VARIANCE OPTICAL COHERENCE TOMOGRAPHY FOR THE PREDICTION OF AGGRESSIVENESS AND RISK OF PROGRESSION OF MELANOMAS

Background

Histology is the gold standard examination for melanoma diagnosis and provides important data for the staging of the tumor, as tumor thickness and presence of ulceration and mitosis rate. Some other exams, such as sentinel lymph node biopsy and Radiology imaging, may be necessary for the final definitive staging. However, these 3 complements modalities of investigation are expensive and may delay the beginning of the therapy. Reflectance confocal microscopy (RCM) is a noninvasive imaging technique, and which has already proved to be able to diagnose and correlate the morphology of the atypical cells with melanoma behavior. Speckle-variance optical coherence tomography (SV-OCT) is a novel in vivo imaging technique that generates images of the skin micro-angiography in transversal and enface/horizontal view. Changes in the skin micro vascularization has been seen with this technique when comparing normal from skin tumors, different skin tumors among them and when comparing melanomas with different Breslow thickness, the last presenting a vascular implement in accordance to the thickness of the melanoma during.

Objectives

Association between RCM features and SV-OCT micro-vascularization analysis for the evaluation of progression and aggressiveness of melanomas. The benefit of this association may represent a future advantage for in vivo staging of this skin cancer, which may lead to a reduction on late diagnosis and implement in patient's targeted approaches, with a final benefit in prognosis and quality of life.

Methods

Eighty lesions suspicious or consistent for melanoma diagnosis upon dermoscopy were evaluated by RCM and SV-OCT, prior to excision. In order to quantify the tumor rate of growth, during the evaluations patients informed since how long the lesions have been there. Once RCM confirmed the in vivo diagnosis of melanoma, a morphologic classification of the predominant atypical cell-types has been done as well as the description of RCM features previously proposed by Pellacani et al. Upon SV-OCT, an evaluation of the tumor micro-vascularization was done in the enface/horizontal view in 2 different depths (150 and 300 μm) The morphology of the vessels (dotts, blobs, coils, lines, curves and serpiginous) and the presence of branching (and its classification as arborizing or bulging)

were evaluated. To consider a certain vascular morphology as present, it had to be seen at least 3 times independently in each depth.
According to histologic descriptors and clinical indication, immunohistochemical markers related to tumor aggressiveness (Ki67, CD271, HIF, -1aCD31) and research of BRAF, NRAS and c-Kit mutations will be performed in a subset of cases. Lymph node biopsy and radiologic imaging were performed for the staging of the tumor. Moreover, sentinel lymph node biopsy and radiologic imaging were performed when indicated.
RCM and SV-OCT evaluations were then correlated with melanoma stages, tumor growth pattern, the progression of the disease (in cases when it was possible to make a follow-up of the patients) and the biomolecular profile.

Results

In the first part of the study there was obtained a correlation between the vascular morphologies seen upon SV-OCT and the Breslow thickness of melanomas. Dotted vessels were frequently seen in both depths and independently from tumor thickness, but the irregular distribution of those vessels was an indicator for lesions thicker than 1mm.
At 150 µm the irregular distribution of dots and the presence of curved vessels were predominant on Breslow > 1mm, whereas coiled and serpiginous vessels were almost only present on Breslow > 2mm. At that depth, bulging branches were only present in melanomas thicker than 2mm.
At 300 µm, the irregular distribution of dots and the presence of serpiginous vessels and bulging branches were predominant on melanomas with Breslow thickness > 2mm.
The correlation of this first part of the results with the RCM findings and immunohistochemical markers are still in progress.

Conclusions

Still in progress.

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AUTOFLUORESCENCE OF SOFT AND HARD HUMAN ORAL TISSUES PRELIMINARY RESULTS FROM DYSPLASTIC, MALIGNANT LESIONS AND OSTEONECROSIS OF THE JAWS

Background

Fluorescence is the property of some substances to emit light at frequency which is different from that of the exciting radiation. Such a phenomenon is due to the presence of endogenous or exogenous molecules (fluorophores), which absorb light energy with a specific wavelength and emit luminescent radiation with a different one.

Endogenous fluorophores are normally present in tissues being related to the phenomenon of Auto-Fluorescence (AF).

AF is a peculiar characteristic of some tissues and depends on the presence and concentration of active fluorophores.

It has been hypothesized that fluorophores of oral human tissues are some proteins (e.g. structural proteins: collagen, elastin; coat proteins: keratin) and several co-enzymes involved in cellular metabolism (NADH, FAD). Such molecules are stimulated by wavelengths between blue and violet / ultraviolet light.

Healthy oral mucosa emits fluorescence, detectable as green light, following stimulation with a monochromatic source of wavelength. In many cases of dysplastic and malignant lesions fluorescence intensity decreases up to disappearance, according to the progression of histopathological changes. Tissues that have lost the ability to emit fluorescence appear dark brown / black. Despite the above mentioned physical observation, controversy exists on the pathobiological mechanism leading to loss of autofluorescence.

Apart from AF of oral soft tissues, fluorescence of healthy jawbone tissue has recently been described. Viable bone appears hyper-fluorescent when irradiated by ultraviolet/blue light, whereas necrotic bone loses such AF, appearing very dark.

The use of endogenous AF of the bone as a possible guide to visualize necrotic bone during surgical debridement/resection has been proposed.

However, to the best of our knowledge no sensible hypotheses to explain loss of AF (LAF) of the necrotic bone tissue have been proposed. Comparative studies are necessary in order to establish a more accurate correlation between bone AF and bone vitality

Objectives

1. Investigate the correlation between degree of AF and histopathological features of oral mucosa, in order to assess the usefulness of AF in the diagnosis of malignant and potentially malignant lesions.

2.Investigate the correlation between degree of AF and histopathological features of jawbone tissue, in order to assess the usefulness of AF in highlighting surgical margins of osteonecrosis od the jaws (ONJ).

Methods

Oral mucosa

Thirty-two lesions suspicious for Oral Epithelial Displasia (OED) and Oral Squamous Cell Carcinoma (OSCC) were included in the present evaluation. Specimens were collected from 14 patients (12 females; 2 males).

The VELscope™ (LED Medical Diagnostics Inc., Barnaby, Canada; 410-430 nm) system was used for stimulating AF of oral mucosa. Each specimen was classified as normo-/hypo-/hyper-/ fluorescent and the histopathological pattern was analyzed.

Jawbone tissue

Eight cases of Medication Related Osteonecrosis of the Jaws (MRONJ) associated to the use of bisphosphonates were included in the present evaluation.

The VELscope™ system was used for stimulate AF of bone tissues. All hypo-fluorescent bone was resected and analysed. Specimens of hyper-fluorescent bone of the surgical field were collected and analyzed in each patient. Specimens were collected from 8 patients (6 females; 2 males).

Results

Oral mucosa

Thirteen (40.6%) out of 32 lesions were classified as hyper-fluorescent. Of these, 9 (69.23%) were OED or OSCC, whereas 4 (38.5%) were non-dysplastic lesion.

Nineteen (59.4%) out of 32 lesions were classified as hypo-florescent. Of these, 18 (94.73%) were OED or OSCC, whereas 1 (5.2%) was non-dysplastic lesion.

Jawbone tissue

All the hypo-fluorescent specimens analysed (8; 100%) were necrotic tissue, whereas all the hyper-fluorescent specimens (14; 100%) were in viable tissue with changes typical of Bisphosphonates (BPs).

Conclusions

Oral mucosa

The preliminary data reported here confirm the hypothesis that both hypo- and hyper-fluorescence are alterations of normal AF and could be in relationship to histopathological alterations such as OED or OSCC.

Research impact: AF could be used to target biopsies in clinical suspect lesions and in highlighting surgical margins during excision o malignant lesions.

Jawbone tissue

Data reported here show a strong correlation between fluorescence of bone and bone viability.

Research impact: Intra-operative identification of necrotic bone is a major difficulty for surgeons. It is usually based on clinical and radiographic parameters. AF could be used in highlighting surgical margins of MRONJ resection.

Future research

In order to establish a more accurate correlation between degree of AF and diagnosis it is necessary to investigate specific histopathological and immunohistochemical markers. Objective measurements of the degree of AF with a more objective methods would be necessary.

Dr. Annalisa Guida

CEM Curriculum: Translational Medicine
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DECIPHERING IMMUNE RESPONSE TO CHECKPOINT INHIBITORS AND FINDING NOVEL BIOMARKERS IN METASTATIC RENAL-CELL CARCINOMA

Background

Nivolumab represents the new second-line treatment for metastatic renal cell carcinoma (mRCC). This drug is a fully human IgG4 against PD-1 and his role is to inhibits programmed death-1 (PD-1)/PD-1 ligand 1 (PD-L1) immune checkpoint. In the majority of patients, this drug is able to restore the patient's tumour-specific T-cell-mediated response thus improving both overall survival and objective response rate. However, a lack of clinical response occurs in a number of patients, which varies according to the drug itself, the underlying disease, and other factors, hence raising questions about how to predict and increase the number of patients who receive long-term clinical benefit from immune checkpoint therapy. Unlike traditional cancer therapies, checkpoint inhibitors act primarily on cells of the immune system. The requirement for the immune system as a mediator of the drug's activity suggests that the balance of positive and negative regulators of the immune response at the time of therapy may be critical for therapy efficacy. Among these regulators, cytokines, chemokines, and other soluble factors regulate the survival, activity, and location of immune cells and thus represent potential players in determining drug efficacy. Of particular interest are soluble factors involved in the recruitment and regulation of effector T cells, the frequency of different subsets of regulatory T cells and the ratio between effector T cells and regulatory T cells.

Objectives

The main aim of this project is to identify immune and serum biomarkers that are modulated in patients with metastatic renal cell carcinoma during and treated with immune checkpoint inhibitors and that can discriminate patients who most likely benefit from such therapy.

Methods

This is a prospective, longitudinal, interventional study on patients with mRCC who will receive Nivolumab in standard clinical practice. The project investigates changes in main immune parameters in patients with mRCC treated with nivolumab by analysing blood samples at baseline and after 1, 2, 3, 6 and eventually 12 months. At each timepoint the following parameters will be evaluated: 1) the frequency and distribution of Teff cells and Treg cells; 2) survival anergy induction and proliferation capacity of Teff cells; 3) polyfunctionality of Teff and Treg cells in response to different stimuli and recall antigens 4) the copy number of Tcell receptor rearrangement excision circles (sjTREC) which is an indicator of thymic functionality; 5) plasma levels of cytokines chemokines and other soluble factors which are involved in the survival activity and trafficking of T

cells. We planned to enrol 30 patients.

Results

From January 2016 until March 2017 we enrolled 10 patients. The median age was 55 years (33-71). The majority of patients had clear cell histology (90%). Nivolumab was given as second-line therapy in 50% of patients, as third line therapy in 40% of cases. According with both Memorial Sloan Kettering Cancer Center Score (MSKCC Score) and International Metastatic Renal Cell Carcinoma Database Consortium Score (IMDC score) 75% of patients are in the intermediate prognostic risk group. The number of metastatic sites involved was: 1 in 20% of patients, 2 in 40% of cases while more than 2 sites are reported in the rest 40% of patients. Eight patients were still ongoing treatment and two patients died due to rapidly progressive disease.

Conclusions

We expect to identify easily measurable immune biomarkers that predict the responsiveness to nivolumab. Finding novel biomarkers that predict the response to therapy with nivolumab and monitor its efficacy can be of great benefit for the success of treatment not only to increase the number of patients who assume this therapy but also to identify those who have to change treatment without losing time so allowing an optimal allocation of economic resources**.** Longer follow up is required to assess preliminary immunological data.

Dr. Eric MARCH

CEM Curriculum: Medicinal and Pharmaceutical Science
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INTEGRATED LIGAND- AND STRUCTURE –BASED APPROACHES FOR PREDICTING COMPOUND POLYPHARMACOLOGY BASED ON BIG DATA

Background

It has always been of primary importance to understand how drugs interact with their targets to properly explain their function. Nowadays, the magic bullet approach, i.e., a drug with high potency and selectivity for a specific target, has become obsolete, mainly because of the appearance of the polypharmacology approach. Polypharmacology proposes that more effective drugs can be developed by a selective modulation of multiple targets (1). This turns out to be a very interesting approach when combined with Big Data, since we can apply multitasking learning algorithms to build polypharmacology prediction models using all the data collected (2). Data includes protein structures, drugs, ligands, drug-target and drug- disease associations made available in public repositories.

Objectives

The main goal of the project is to develop a computational tool to detect, describe and compare protein target binding sites, both from a structural and a ligand point of view. This tool will ultimately be used to predict polypharmacology of different compounds chosen by the user.

Methods

We downloaded from PDB (3) a set of kinases structures, all belonging to Cdk2 family, to assert the robustness of the procedure.
Using fpocket software (4), we detected and retrieved several pockets for each protein, from which we identified their orthosteric binding site thanks to the fpocket scoring scheme. From there, we generated the surface for each protein using MSMS (5). Then, we kept the part related to the binding site and we calculated descriptors encoding properties of protein surface residues following the method of Cipriano et al. (6).
Once the data was collected, we repeated the same procedure with different protein families such as GPCRs and bromodomains. To analyze the data, the different data sets of protein descriptors were analyzed using Principal Components Analysis (PCA) and hierarchical clustering. A set of classes were obtained and used to visualize the different patches in the binding site surface. This allowed us to map the pocket by its protein surface residues and identify common regions between binding sites of different proteins.

Results

We obtained a set of surface representations of the protein binding site.

For every point in the surface, we obtained a score after applying PCA and hierarchical clustering that allowed us to "color" the surface based on different patches. Each color refers to a different property encoded by the residues in the protein. So for each pocket we were able to highlight regions of the surface with similar properties.
Using these patches, we compared the different binding pockets, in order to see if there are patches that match in similar regions of both pockets.

Conclusions

Starting from the protein structures available in the PDB, we have started developing and testing a rigorous method to identify, describe and compare the binding sites of different proteins.
The studies conducted so far show that the PCA analysis highlight regions of the binding site surface with similar properties, allowing the identification of patches and comparison of binding pockets. The analyses are based on a limited set of proteins and substantial more work will be needed to assess the validity of the proposed approach.
Our main interest is to integrate this information with ligand-based approaches to detect polypharmacology of the desired compounds and ultimately generate a multi-tasking model to assess these predictions. Finally, a web server will be created so users could upload and work with their own data. If new targets are identified, these findings could lead also to drug repurposing.

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NON-INVASIVE PREDICTION OF NONALCOHOLIC STEATOHEPATITIS (NASH) THROUGH COMBINATION OF ULTRASONOGRAPHIC, ANTHROPOMETRIC AND METABOLIC INDICES. CORRELATION BETWEEN ULTRASONOGRAPHIC AND CARDIOVASCULAR PARAMETERS

Background

Nonalcoholic fatty liver disease (NAFLD) affects up to one-third of the general population in Europe and USA and two-thirds of patients with obesity or type 2 diabetes, but this prevalence is probably underestimated, given the lack of a highly reliable non-invasive diagnostic test (Barb D., Metabolism Journal 2016). The natural course of NAFLD begins with simple steatosis which, in a fraction of cases, may progress to NASH (nonalcoholic steatohepatitis). NASH is defined by ballooning degeneration, and inflammatory-fibrotic changes and has the potential to progress to cirrhosis and even hepatocellular carcinoma (HCC) (Marrero JA, Hepatology 2002).

NASH is now the second cause of liver transplantation in the US (Rinella ME, JAMA 2015). However premature mortality in nonalcoholic steatohepatitis is related to both hepatic (cirrhosis and HCC) and extra-hepatic complications, chiefly cardiovascular disease. Indeed NAFLD is a systemic condition characterized by hepatic and peripheral insulin resistance with fasting hyperglycemia and compensatory hyperinsulinemia which can, over time, lead to type 2 diabetes mellitus, endothelial dysfunction, dyslipidemia and premature atherosclerotic cardiovascular disease (Koh KK, J Am CollCardiol. 2005). Therefore, prompt recognition of progressive liver disease is key in order start an aggressive management (intensive life-style changes) is key to prevent hepatocellular injury and cardiovascular complications. Nowadays hepatic biopsy is the only diagnostic test, but it is an invasive procedure and has limitations and risks.

Objectives

This study aims to identify the independent predictors of NASH and hepatic fibrosis, by elaborating a diagnostic algorithm which combines anthropometric, ultrasonographic, elastography and metabolic parameters. Furthermore, the study includes a follow-up of hepatic, metabolic and cardiovascular complications.

Methods

In November 2016 a prospective observational study began and it is still ongoing. Criteria for inclusion: patients with a diagnosis of NAFLD or suspected NASH, from the participating centers [outpatient clinics and inpatient wards in Department of Metabolic Medicine of Baggiovara Hospital (Modena) and Internal Medicine of Pavullo Hospital (Modena)]. The diagnosis of hepatic steatosis was based on ultrasonographic findings and NASH suspected based on altered laboratory or transient elastography tests.

Criteria for exclusion: presence of any of secondary causes of hepatic steatosis: (Chalasani N., Gastroenterology 2012), (alcoholic, viral, autoimmune, genetic, or drug-induced), pregnancy. Excessive alcohol consumption was defined as >30 g/day for men and >20 g/day for women). Hepatitis B was excluded by doing total anti-HBc, anti-HBe and HBsAg. Hepatitis C Virus infection was excluded by anti HCV negativity. Antinuclear antibodies, immunoglobulin G, iron studies, ceruloplasmin levels were done to rule out other etiologies of hepatic disease. Metabolic syndrome was diagnosed according to National Cholesterol Education Program Adult Treatment Plan III (NCEP-ATP III) guidelines (Grundy SM, Circulation 2004). Diabetes mellitus was diagnosed based on American Diabetes Association criteria (ADA, Diabetes Care 2006) or on patients being under treatment with oral hypoglycemic agents. A family and personal history was collected in each patient. During the interview, patients were inquired for comorbidity and medications, eating habits and physical activity, using validated questionnaires such as food diary and international Physical Activity Questionnaire (iPAQ) short form. Obesity was defined as BMI cut-offs of 25 and 30 kg/m-2 for overweight and obesity, respectively, according with the World Health Organization (WHO). Complete laboratory panel, liver tests and lipid profile were done at the time of enrollment unless performed in the last month. Homeostasis Model Assessment index for insulin resistance (HOMA-IR) was calculated by the following equation: fasting blood glucose (mg/dl) x fasting blood insulin (mcU/ml)/405 (Matthews DR, Diabetologia 1985). A ultrasound (US) scan of abdomen was executed to ascertain prevalence and severity of hepatic steatosis, using US-FLI a validated semi-quantitative score for fatty liver (Ballestri S, Liver Int 2012). US scan was used also to evaluate visceral adipose tissue (VAT) and subcutaneous adipose tissue (SAT) (Pontiroli AE,

ObesSurg 2002). Transient elastography scan (Fibroscan) was performed to estimate Controlled Attenuation Parameter (CAP) and liver stiffness (LS) (Nascimbeni F, Clin Gastroenterol Hepatol. 2014; Ballestri S, Expert Rev GastroenterolHepatol 2015). Only in selected patient was liver biopsy performed, after obtaining informed consent. All biopsies were read by our liver histo-pathologist. Histological features were evaluated using NAFLD activity score (NAS) based on steatosis, ballooning and inflammation (Brunt et al, Am J Gastroenterology 1999). The study was approved by the Institutional Ethics Committee of Modena. A written informed consent was taken from each patient at the time of enrolment.

Results

As of March 2017, 40 subjects older than 18, with an ultrasonographic diagnosis of fatty liver, have been included. They were all obese with a BMI > 30 in 90% of cases; 95% were dyslipidemic with: a mean total cholesterol of 216 mg/dl (min 147-max 363), a mean LDL cholesterol of 140 mg/dl (min 90-max 194), a mean HDL cholesterol of 50 mg/dl (min 33- max 84), a mean triglyceride of 178 mg/dl (min 52- 1429 max). Patients had, in 37,5% of cases, a cardiovascular disease (hypertension) and in 35% ones, an altered glycemic profile, with a diagnosis of Impaired Fasting Glucose (IFG) or Diabetes Mellitus (DM). 85% of patients had altered liver enzymes.

Among evaluated patients, only 9 were conducted to hepatic biopsy as their condition was strongly suspected for progressive liver disease. Mean age was 51,5 years with an equal division between male and female, but female were older than male. All women were in menopausal state. 33% of biopsied patients had a BMI ranging from 23 to 26 (normal weight). The remaining ones had a BMI > 30, with a mean waist of 110 cm and mean hip of 113 cm. In the 89% of cases they had a previous persistent elevated aminotransferase levels since at least 2 years. Hepatic steatosis was evaluated with US-FLI score and transient elastography. US-FLI was > 4, and patients showed a mean Liver Stiffness of 7.9 kPa (min 5.1 – 20.6 max), and a mean CAP of 321 db/min (min 252- 376 max). 44% had a cardiovascular disease or altered glycemic profile. All biopsied patients were dyslipidemic, with a mixed dyslipidemia in 55,5% of cases. Histological analysis revealed NASH in 89% of biopsies; only one was simple steatosis. Among NASH cases 5 were stage 1; 2 were stage 3; 1 was stage 2. The patient with simple steatosis was: a normal-weight (BMI 23) young man, who had dyslipidemia, a persistent hypertransamisemia, high GGT level, US-FLI score of 4/8, at transient elastography scan a Liver Stiffness of 6,1 and a CAP of 252. He had a family history for metabolic disease (DMT and hypercholesterolemia) but he hadn’t altered glycemic profile or any cardiovascular disease. The two patients (a man and a woman) with the highest

histologic stage score (3) were obese, with a BMI > 31 and a waist circumference > of 107 cm. The man had US-FLI score of 7, a Liver Stiffness 5,5 kPa and a CAP of 376 db/min, and a persistent hypertransaminasemia. The woman had a lower instrumental score (US-FLI score 5, LS 5,1 kPa, CAP 345 db/min), and she had a diagnosis of IFG.

Conclusions

In this study we compared histologic results with laboratory data in order to verify diagnostic performance of ultrasonographic, elastographic and clinical anthropometric data. Preliminary analysis supports a correlation between clinical-laboratory data and histological results. All selected patients, on the basis of clinical and instrumental characteristics, had a diagnosis of progressive liver disease. Most of them had metabolic syndrome.

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CEM Curriculum: Medical and Pharmaceutical Sciences
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BBB-TARGETED PIC MICELLES FOR DRUG AND ROS RESPONSIVE GENE MATERIAL DELIVERY FOR THE TREATMENT OF BRAIN PATHOLOGIES

Background

Nowadays, there is a lack of efficient therapeutics to treat Central Nervous System (CNS) pathologies such as Alzheimer's disease (AD), Parkinson's disease (PD) and brain cancer. This is due to the impairment of actual therapeutics to cross the Blood Brain Barrier (BBB), which is the main barrier to reach the brain. The development of nanosized drug delivery systems that cross the BBB and transport therapeutics to the CNS is mandatory to resolve this issue. Our research group developed PLGA nanoparticles targeting the BBB through the surface engineering with glycopeptide (g7) and demonstrated that these g7-NPs cross BBB after systemic *in vivo* administration in rodents and to be able to deliver both low and high molecular weights therapeutics to the brain[1–3]

Currently, there are a number of studies on gene material delivery for the treatment of brain specific pathologies [4,5]. Main limitations in gene material delivery consist of its instability when administered systemically and by its inability in being transferred to a specific cell population. Thus, the development of targeted nanosystems able to protect this genetic material from degradation as well as from being recognized by the immune system is most than welcome. Polyion Complex Micelles (PIC micelles) are core-shell structures formed by the ionic interaction between a block copolymer and counter charged compounds[6]. Gene material loaded PIC micelles can be prepared by complexion of gene material conjugated Polyethylenglicol (PEG) (a neutral, hydrophilic and biocompatible polymer) blocks with positive charged polymers. The aforementioned issues on gene material delivery can be overcome due to the high steric repulsion effect of PEG shell and the effective shielding of the internal cargo from external hazards. As many pathological conditions, including those of the CNS, are associated with elevated levels of reactive oxygen species (ROS), a possible goal is to plan nanosystems with the ability to respond to this bio-signals for the selective release of therapeutics in such target sites[6]. The aim of this PhD project is to develop BBB targeted PIC micelles for drug delivery as well as ROS responsive gene material delivery for the treatment of CNS diseases. Cationic polymers as polyamidoamine dendrimers and

gene material conjugated, via a Thioketal (TK) bond, to g7-PEG, are going to be used for PIC micelle formation.

Objectives

The aims of the PhD project are to develop BBB-crossing and ROS-responsive PIC micelles for the delivery of gene materials. The first specific objective was to synthesize and characterize the Thioketal-containing linker, to prepare mPEG-TK by conjugating covalently mPEG-NH2 to this molecule and finally to verify that under ROS conditions, a cleavage of mPEG-TK bond happens. With this first aim accomplished, the subsequent main objectives that are the conjugation of mPEG-TK to gene material and PIC micelle formation by complexion with a polycation polymer, can be done. The physical and chemical properties of this micelles as well as their therapeutic activity on ROS-producing cell cultures, are going to be evaluated before studying g7- modified PIC micelles.

Methods

Protocols used to synthesize TK-containing linker (HOOC-TK-COOH), previously described by Li et al., 2016[7,8] and Yue et al., 2016[9], were slightly modified and applied. Briefly, a mixture of 3-mercaptopropionic acid and anhydrous acetone (1:2 ratio) was saturated with dry hydrogen chloride and stirred at room temperature for 4 h. Thereafter, the reaction was quenched by placing the mixture on an ice bath until crystallization. After washing the crystals thoroughly with hexane and cold water, the white powder obtained was characterized by ^{1}H NMR in DMSO-d6 and CDCl3. For TK-containing linker conjugation to mPEG-NH2, a 10 to 1 molar ratio of these reagents was used and mixed in dichloromethane (DCM) with equal moles of EDC-HCl and NHS. This mixture was stirred for 48 h and after evaporating the solvent, the product was precipitated with cold ether and purified by dialysis and characterized by ^{1}H NMR in CDCl3. The breakage of mPEG-TK bond is going to be evaluated by its incubation under ROS conditions and assessing by ^{1}H NMR the signal disappearance due to TK protons. This study is going to be done before the conjugation of mPEG-TK-COOH with gene material and the subsequent formation of PIC micelles. The size and morphology of PIC micelles are going to be studied as well as its therapeutic activity on cell cultures.

Results

The synthesis of TK- containing linker was accomplished with experimental yield (25%) and characterized by ^{1}H NMR in DMSO-d6 and CDCl3. The ^{1}H NMR spectrum in DMSO-d6 depicted three signals (expressed in parts per million) corresponding to TK- containing linker (previously

reported by Yue et al., 2016[9]): 2.74 ppm (t, 4H), 2.50 ppm (t, 4H), 1.53 ppm (s, 6H). A ^{1}H NMR in CDCl3 was also done, obtaining the following relevant signals: 2.93 ppm (t, 4H), 2.70 ppm (t, 4H), 1.62 ppm (s, 6H).

Conclusions

In this first part of the PhD, we can conclude that the ROS responsive molecule, TK- containing linker, was successfully obtained and characterized. Currently, we are working in obtaining the conjugate mPEG-TK-COOH which is meaningful for proceeding with the subsequent objectives of the project as mPEG-TK-COOH conjugation to gene material and PIC micelle formation.

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CEM Curriculum: Translational Medicine
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ANAEMIA AS NEGATIVE PREDICTIVE FACTOR FOR TUMOR RESPONSE TO NEOADJUVANT CHEMOTHERAPY IN EARLY BREAST CANCER PATIENTS

Background

Neoadjuvant systemic therapy (NST) is a treatment option in patients with early-stage breast cancer (BC). Tumor response to NST well correlates with survival, and pathological complete response (pCR) significantly predicts long-term outcomes. Anaemia is one of the most common side effects of cytotoxic drugs. Biologically, anaemia produces low intra-tumoral oxygen levels that seem to induce many adaptive responses in cancer cells such as overexpression of hypoxia inducible factor-1 (HIF-1), epidermal growth factor receptor (EGFR) and vascular epidermal growth factor (VEGF). These biological modifications could be responsible for increase in chemo-resistance. In literature, data on the predictive role of anaemia and hypoxia induced by chemotherapy during primary treatment in BC patients are lacking. We have hypothesized that the grade and the duration of anaemia could represent a negative predictive factor for tumor response.

Objectives

The aim of the study is to evaluate the influence of Hb level throughout treatment course on tumour shrinkage induced by NST. Moreover, we want to investigate the relationship between anaemia and the expression of hypoxia-related biomarkers and genes in anaemic women with residual BC disease after NST.

Methods

317 patients diagnosed with stage I-III BC treated with primary chemotherapy were evaluated. Patient and tumor characteristics and treatment information were collected. Standard biological

parameters *(*Ki67, nuclear grade, hormone receptors and HER2 status*)* were correlated to pCR. We focused on Hb level (at baseline, at the end of NST, drop in Hb throughout treatment, duration of anaemia) and its correlation with the pCR rate. Anaemia was defined as a drop of Hb under the local limit of normal in women (12 mg/dl). The next step will be the analysis of HIF-1, VEGF and EGFR expression and their correlation with pCR and survival outcomes in patients with residual disease after NST. Finally, we will analysis the expression of hypoxia-related genes in the same subgroup of patients.

Results

Globally, pCR was achieved in 83 patients (26%), mainly HER2 positive disease (Hormonal receptors positive/HER2 negative = 6%, HER2 positive = 41%, triple negative = 37%; p<0,0001). Median baseline Hb was 13.3 g/dl while median Hb level at the end of NST was 10 g/dl. pCR rate was not influenced by baseline Hb level. Anaemia due to chemotherapy was reported in 60% of patients. No difference in Hb levels was observed stratifying patients according to nuclear grade, tumour stage and cancer subtypes. Anaemia at the end of NST was an independent negative predictive factor for pCR in univariate and multivariate analysis (p=0.009). In the subgroup of anaemic patients the *decrease in Hb* ≥ 2 *g/dl* from baseline was associated with a significantly lower rate of pCR (15% vs 28%, p=0.009). Moreover, in anaemic patients with duration of anaemia longer than two months and *decrease in Hb* ≥ 2 *g/dl the rate of pCR was the lowest (10%, p=0.01). The evaluation of* expression of HIF-1, VEGF and EGRF and hypoxia-related genes are ongoing.

Conclusion

Preliminary results show an independent negative predictive role of anaemia in women treated with NST for BC. This evidence suggest that anaemia should be corrected in order to obtain the best response to primary treatments.

Dr. Antonio Quotadamo

CEM Curriculum: Medicinal and Pharmaceutical Sciences
Tutor: Prof. Maria Paola Costi

SYNTHESIS AND BIOLOGICAL EVALUATION OF NOVEL PROTEIN-PROTEIN INTERACTION INHIBITORS OF THYMIDYLATE SYNTHASE AGAINST COLORECTAL CANCER

Background

Colorectal cancer (CRC) is one of the major causes of mortality throughout the world and it is the third most common form of cancer with 610.000 deaths per year. The treatment is mainly based on chemotherapy, employing 5-FU, Capecitabine and Raltitrexed as first line drugs. These antimetabolites, target the human Thymidylate synthase (hTS), a key homodimeric enzyme involved in the synthesis of DNA. As a consequence, hTS is a target for anticancer chemotherapy by several drugs in treatment of colorectal and other tumours. While the catalytic activity is specific of the homodimeric conformation, the monomers of hTS can bind to its mRNA controlling the levels of enzyme expression by repressing its translational efficiency[1]. The presence of excess substrate or inhibitors of TS stabilize the homodimeric conformation leading to decrease its binding to mRNA, resulting in increased translational efficiency and ultimately increased levels of TS protein. hTS overexpression due to increased gene transcription and mRNA translation can mediate toxicity and drug resistance. Moreover decreased cellular uptake and polyglutamylation of TS-targeting drugs (raltitrexed), increased drug efflux, altered metabolism of cytotoxic drugs (5-FU) and other intracellular events can decrease the effectiveness of TS-targeting drugs.

Objectives

The aim of my PhD project is to develop new dissociative inhibitors able to induce a perturbation in the dimer-monomer equilibrium in favour of the monomeric form, thus inhibiting the catalytic function and preserving the regulatory activity. With this purpose, my work is specifically focused on the design, synthesis, characterization and evaluation of the enzymatic and cellular inhibitory activity of new compounds, potentially endowed by dissociative mechanism [2].

Methods

Compounds synthesis: Different compounds have been processed by means of specific reaction steps whose number strictly depended on their retrosynthetic analysis, using conventional or microwave-assisted synthesis by comparing total reaction time and percentage yield. Different

experimental conditions have been taken into account to establish the synthetic and analytical procedure for the new compounds.

The single intermediates and the final compounds were purified by crystallization or chromatographic techniques. The chromatography process was carried out either with ISOLERA (Biotage® automatic purification system) or using the flash column chromatography, with regards to the purification complexity of the newly obtained molecular candidates. The compounds were fully characterized through Nuclear Magnetic Resonance (NMR) spectroscopy, mass spectral techniques (Ion Trap LC-MS) and elemental analysis.

NMR spectra (1D-NMR: ^{1}H and^{13}C-NMR; 2D-NMR: COSY, HSQC, and HMQC) were recorded on a *Bruker Advance 400 MHz WB* spectrometer operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C.

Biological evaluation: Enzymatic inhibition assays towards the target (hTS) and MTT assays for anticancer activities towards HT29 and A2780/CP were performed at Drug Discovery and Biotechnology Lab - University of Modena and Reggio Emilia (Italy).

Results

In the first part of my PhD project, based on a mixed ligand-based and structure-based approach, I focused on the design and synthesis of 15 new derivatives, analogues of a HIT compounds (LC1343, LC1273) obtained from tethering approach in previous studies, with the intent to improve the dissociative-inhibitor activity and the biological features. Moreover LC1343 was re-synthetized in high quantitative in order to study the activity in formulation due to the poor drug solubility and to confirm that the decrease in TS levels seems to be ascribable to a reduced enzyme stability and an increased degradation by proteasome. So, it is evident that our novel inhibitors act differently from classical TS inhibitors at a cellular level.

Aiming to explore benzothiazole moiety present in LC1343, seven new benzothiazole derivatives have been synthesized in order to make them in peptide coupling reaction to produce new final compounds.

A validated analytical method using automatic purification system in high peak resolution was achieved. In details, 5 of these compounds present an IC_{50} below 30 µM with uncorrelated FRET value due to the different of test concentration in preliminary FRET screening at 10 µM. Among all the 15 final compounds synthetized, 9 of them have been tested against the 2 cell lines with a cellular death around 20-30% at 40 µM. An important goal was obtained with compound C5, showing a good anticancer activity with 68% of cell growth inhibition against a 5FU resistant, HT29 cell line.

Conclusions

On the basis of the obtained data from the hTS enzyme, specifically related to FRET and cell growth in MTT assays, we have selected the most promising compounds for continuous structure activity relationship (SAR) study in order to found a lead candidate. According to literature the compound survey studies indicate that PPI inhibitors tend to be more hydrophobic, more rigid, and contain multiple aromatic rings; these characteristics however, directly translate in a reduced solubility for enzymatic assays due to the lipophilic group. For this reason, the forthcoming compounds will include a polar group to balance the LogP value and PPI activity of compounds. Such results demonstrate that it was possible to identify new promising compounds, representing novel therapeutic agents acting as dissociative inhibitors of hTS, with the peculiarity to avoid target protein overexpression.

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Acknowledgement

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Dr. Angelo Territo

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CoTutors: Dr. Alberto Breda, Prof. Salvatore Micali

ROBOTIC KIDNEY TRANSPLANTATION: EUROPEAN ONE-YEAR DATA

Background
Kidney transplantation (KT) is the preferred treatment for patients with end-stage renal disease. In order to reduce the morbidity of the open surgery, a robotic assisted approach has been recently introduced. According to the literature, the robotic surgery allows the performance of KT under optimal operative conditions while maintaining the safety and the functional results of the open approach.

Objectives
We present the one-year results from the ERUS Robotic Kidney Transplantation Group common prospective recruitment database of Robotic assisted kidney transplants (RAKT) on 96 cases.

Methods
An ERUS RAKT group was created in July 2015 with the intent of generating prospective data on robotic kidney transplantation. A common prospective recruitment database of RAKT performed at 8 different European Centers was therefore created in July 2015. Functional and surgical data were analyzed and herein reported.

Results
The patients demographic characteristics were as follows: 36 adult females and 60 males with mean age 41.6 years old (range: 18-63), mean BMI 25,5 kg/m2 (range: 17-37), and mean pre-transplantation serum creatinine 570.8 µmol/L (range:198 - 1414) with a mean GFR: 11.3 ml/min per 1.73 m2 (range: 3-23). There were no vascular and ureteral anomalies in the cases included. The mean ASA score were 2. Overall surgical time was 268 min (range: 160 - 430) with vascular suture time of 41 min (range: 24 - 85), and estimated blood loss 145 ml (range: 40-400 ml). Overall ischemia time (including warm ischemia, cold ischemia and rewarming time) was 110 min (range: 58 – 377). The average rewarming time was 55 min (range 34 – 110). Two patients were converted to open transplantation. No major surgical intra- operative complications were

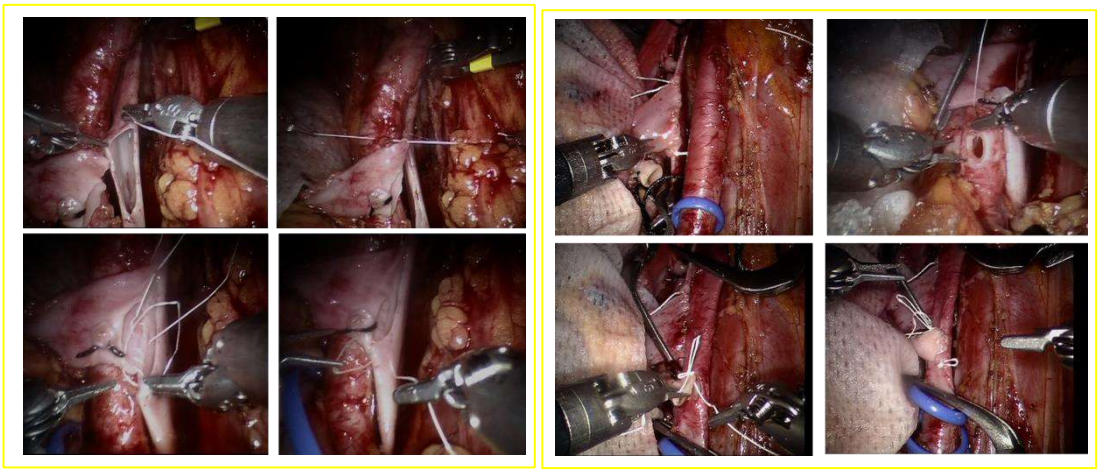
observed. There were three cases of transplantectomy for a massive arterial thrombosis on on post-operative day (POD) 2. One case of intraperitoneal hematoma occurred on POD 1, and was successfully managed laparoscopically. The mean post-operative serum creatinine level was 190 µmol/L (range: 62 – 1326) on POD 7.

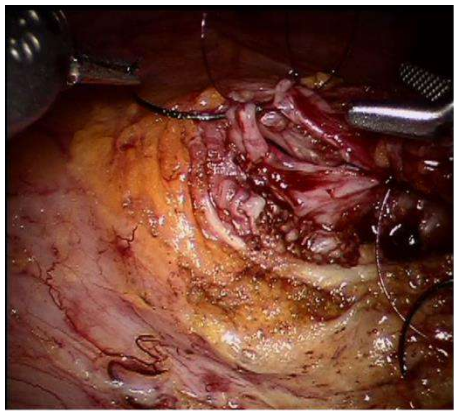
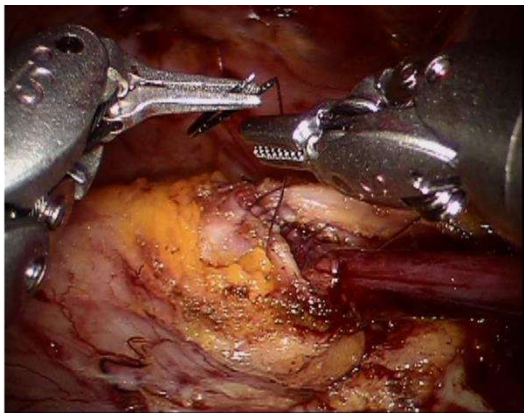
During the course post-operative were observed: one case of lymphocele, two of ileus and one the sepsis.

Post-operative pain, evaluated with Visual Analog Scale (VAS) score, was optimal. The mean hospital stay was 6 ± 1 days (range 4 - 8 days). The mean time of ureteral catheter was 30 days (range: 14 – 110) after the surgery. There were five cases of delayed graft function although at 1 month follow up. Furthermore, no arterial nor ureteral strictures occurred.

Conclusions

This is the first European multicentric study on RAKT. RAKT with regional hypothermia appears to be a safe and reproducible surgical procedure in a properly selected group of patient. The potential advantages of RAKT are related to the quality of the vascular anastomosis, the possible lower complication rate and the shorter recovery of the recipients. The success rate in this group is comparable to conventional open KT.



Dr. Eleonora Truzzi

CEM Curriculum: Medicinal and Pharmaceutical Sciences
Tutor: Prof. Eliana Grazia Leo

DISEGN OF LIPID-BASED PARTICLES AS VESATILE PLATFORM FOR THE DEVELOPMENT OF DELIVERY SYSTEMS FOR DRUGS AND DIAGNOSTICS

Background

The term drug delivery system (DDS) refers to formulations, technologies, and systems to transport drugs and pharmaceutical compounds in the body. DDS offer various advantages respect to conventional administering systems, such as controlled release, increased solubility, protection and targeting of active compounds. Among DDS, lipid-based systems are attractive candidates in this field thanks to their higher degree of biocompatibility and versatility. These systems are commercially viable to formulate pharmaceuticals for topical, oral, pulmonary, intranasal or parenteral delivery.

Nowadays, oral delivery is the most accepted route of drug administration even if it is associated with restraints related to the poor drug bioavailability. Solid Lipid Nanoparticles (SLNs), a lipid-based system, offer advantages such as good tolerability, low drug leakage, high oral drug bioavailability and delivery of drugs into the systemic circulation through the intestinal lymphatic absorption. SLNs promoting the lymphatic absorption can be also exploited for theranostic purposes and this possibility, to our knowledge, has been poor investigated. Theranostics is the fusion of therapeutic and diagnostic approaches that aims to personalize and advance medicine. Magnetic nanoparticles (MNPs) represent a particularly appropriate tool based on their ability to be simultaneously functionalized and guided by external magnetic field. In this field, iron oxide (Fe_3O_4) MNPs provide a unique nanoplatform with controlled sizes and controlled surface chemistry. Without a coating, MNPs have hydrophobic surfaces with a high area to volume ratio and a tendency to agglomerate. Vismara *et al*. proposed in their work the use of heparin as non-covalent coating for iron oxide nanoparticles (Fe@hepa) [1].
Different studies demonstrated that heparin and low molecular weight heparins, in addition to anticoagulant properties, are anti-angiogenic agents and possible vectors to reach tumour sites, due to their ability to bind over-expressed proteins. Thanks to these features, heparin coating specifically directs iron oxide nanoparticles to tumour environments in order to accomplish theranostic aim. However, heparin surface shell is instable in physiologic environment where the

presence of ions reduces the strength of the electrostatic bond between the positive iron oxide core and the negative chain of heparin.

Objectives

Fe@hepa are a promising theranostic tool without any oral *in vivo* application due to its instability. Therefore, to achieve a stabilization of the heparin coating in physiological environment promoting at the same time the oral absorption through the lymphatic route, Fe@hepa were encapsulated in a biocompatible solid lipid shell obtaining Self-Assembled Lipid Nanoparticles (SALNs).

Methods

SALNs were obtained by an original self-emulsification process avoiding the use of organic solvents. The Fe@hepa loaded SALNs (SALNs-Fe@hepa) were characterized about their physical-chemical properties. The particle size, the polydispersity index and the Z-potential value were determined using Photon Correlation Spectroscopy (PCS). SALNs morphological features were analyzed by Scanning Electron Microscopy using both the SEM and the TEM mode. SALNs-Fe@hepa encapsulation efficiency and *in vitro* stability during storage were determined quantifying the amount of Fe@hepa outside the system by a spectrophotometric method based on the formation of highly coloured complexes iron-thiocyanate ions. Finally, the stability of the coating was evaluated quantifying the amount of heparin released in physiological solution using the Azure II colorimetric method. *In vitro* cytotoxicity studies were performed using MTT assay and cell internalization was evaluated in CaCo-2 cell model using both modalities quantifying the amount of Fe@hepa into cellular lysate and observing cells after incubation with labelled samples by confocal laser scanning microscope.

Results

The results revealed that SALNs satisfy the prerequisite to gain intestinal lymphatic absorption. In fact, SALNs-Fe@hepa showed a particle size < 200 nm and a negative Z-potential value. SALNs demonstrated to be an efficient drug carrier since the encapsulation efficiency was higher than 90% with a great stability in suspension. Moreover, SALNs demonstrated to be able to overcome the Fe@hepa instability in biological fluids. In fact, no heparin loss occurs when Fe@hepa are embedded inside the lipid matrix, preserving in this way the theranostic potentiality of the tool.
In vitro studies on CaCo-2 cell model, used as an indirect indication of intestinal lymphatic absorption, showed that SALNs-Fe@hepa are not cytotoxic at the concentrations tested and that

they are able to be internalized by the cells. On the contrary, naked Fe@hepa seem to be not internalized due to its instability in biological fluids since heparin loss provokes the aggregation of iron oxide nanoparticles and their precipitation before that internalization occurs.

Conclusions

SALNs are a drug delivery system that can be exploited for oral administration thanks to their safety, biocompatibility and possibility to achieve intestinal lymphatic absorption. In this work it was demonstrated that SALNs are efficient carrier for Fe@hepa nanoparticles, reducing their cytoxicity on CaCo-2 cells and overcoming the loss in biological fluids of heparin coating.

Future Researches During the next months, different lipid carriers for the delivery of natural compounds by intranasal route for the treatment of neurodegenerative disorders will be developed.

The blood-brain barrier (BBB), which protects the brain and spinal cord from various potential toxic substances and pathogens, also presents a barrier in the treatment of neurodegenerative disorders. Different strategies to overcome the BBB were investigated. Among them, one of the most promising administration routes is the intranasal, since is a non-invasive way to deliver efficiently compounds to the brain.

New engineered drug delivery systems based on lipid hybrid micro-nanoparticles will be designed in order to promote the nose-to-brain transport of potentially active molecules against neurological diseases.

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CEM Curriculum: Medicinal and Pharmaceutical Sciences
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MOLECULAR ASPECTS IN THE SEARCH FOR ANTITUMORAL AND ANTI-INFECTIVE DRUGS

Background

The drug discovery process has been largely improved in the last years thanks to contributions from genomics, proteomics and technological advancements, that have improved both the molecules and the delivery systems. Being able to obtain information regarding the structure of a certain target as well as the molecular details of its interactions with an inhibitor, may constitute a huge input in the drug development process. In this context, this thesis project will evolve around two main research topics.

The first one regards a group of infections known as neglected tropical diseases, and among these, the main objective lies in the search for inhibitors of an enzyme, pteridine reductase 1 (PTR1) – regarded as a mediating target for infections of different pathogens, especially two important parasite familiess, namely Leishmania spp. and Trypanosoma spp. Relevance of PTR1 as a pharmacological target derives from conferring pharmacoresistance to MTX after its overexpression[1], as it salvages oxidized pteridines in Trypanosomids. It also shows an important folate and NADPH-based activity , functionally overlapping other relevant target enzymes that may have been inhibited, such as the dihydrofolate reductase (DHFR). Future endeavours in approaching these pathologies will have to account for such relevant functions expressed by different targets.

The second line of research in this thesis work concerns one of the most important antitumoral targets, human thymidilate synthase (hTS) especially relevant in both the colorectal and the ovarian cancers. hTS is a protein characterized by two linked monomers with an identical primary structure which, in an aqueous solution, equilibrates between the monomeric and the dimeric states. Akin to the PTR1, TS is folate dependent and catalyzes the methylation of 2'- deoxyuridine – 5' – monophosphate or dUMP to 2' – deoxytimidine – 5' – monophosphate or dTMP, using a methyl group given by a cofactor, the N5N10methylentethrahydrofolate. While only the dimeric

enzyme expresses the catalytic function, preliminary work[2] shows that this protein can down-regulate itself. Although the down-regulation mechanism is still being debated, some studies considers only the monomer as capable of such a function. In other words, the higher concentration of hTS monomers in solution, the lesser the mRNA will be translated[1]. Protein over-expression is deemed to represent one of the molecular mechanisms that lead to the known phenomenon of pharmacoresistance in therapeutic protocols that combine platinum-based drugs with classical inhibitors that bind at the catalytic site of the hTS dimer. Therefore, to overcome the onset of resistance, innovative hTS inhibitors that act with molecular mechanisms other than competitive binding are actively searched for.

Objectives

The project's long term objective will delve into the molecular aspects of the inhibition of the two aforementioned targets, hTS and PTR1. On the former, the main objective would be to investigate the molecular mechanistic aspects of some recently discovered allosteric hTS inhibitors[3], thus establishing directions for the improvement of current molecular candidates. This part of the thesis work will be devoted to the investigation of the mechanism of action of candidates that either stabilize the inactive form of the protein[5] or destabilize its dimeric assembly, thus acting as dissociative inhibitors[4]. Regarding PTR1, the main objective lies in the expansion of current knowledge on the molecular mechanistic aspects of the
activity of the main lead compounds, also focusing on their ability to bind other possible targets (dual or multiple inhibitors).

The first year of my PhD work will be focused on delving the current methods and protocols to establish my know-how, and, at the same time, develop and test new methods and modifications of the existing procedures able to introduce significant efficiency improvements. Furthermore, I will contribute to the investigations of the mechanisms of action of our drug candidates at the molecular level, including their interactions with mutated targets. This work aims at improving both the testing of drug candidates and the structural interpretation of their activity, thus forming bases for discovering structure-activity relationships able to suggest rational directions for chemical modifications of the current candidates.

Methods

To achieve the aforementioned results spectroscopic techniques, including UV-vis, circular dichroism and stationary and time–resolved fluorescence spectroscopies, will be employed, together with such other biophysical tools as isothermal titration calorimetry. Fluorescence microscopy will be used to investigate processes relevant to the objectives occurring in cells, e.g., the localization of drug candidates and their effects on the dimeric assembly of hTS.

In the first few months, the first task has been to express and purify both the wild type and mutant hTS enzymes. The purification process was established on the basis of a MPLC system, the AKTA Prime. To assess functionality of the purified enzymes, a spectrophotometric kinetic assay was employed (Beckman Coulter DU – 640 UV-Vis spectrophotometer). Fluorescence spectroscopy has been employed to follow hTS folding and unfolding in the absence and presence of some dissociative inhibitors. FRET - Förster Resonance Energy Transfer – based experiments were performed to quantitatively define the equilibrium between the monomeric and the dimeric assemblies of hTS in the presence of candidate dissociative inhibitors.

Results

- Achieved results: these regard mostly the expression and purification of WT and mutant hTS proteins, which are necessary to the forthcoming experiments.
- Expected results: given the amount of target proteins on one side – especially mutants – and dissociative inhibitors on the other, the expected result of all experiments would be a panel of analyzed data which will work in two different directions: it will serve as a base for improving existing molecular candidates and for tuning current protocols.

Conclusions

In conclusion, the thesis project as a whole will delve into the molecular aspects of the interactions between both WT and mutant hTS enzymes and some recently discovered protein – protein interaction disruptors, as well as between the PTR1 enzymes and their inhibitors. The first year of this PhD work will be focused on the improvement of the currently employed experimental, mainly spectroscopic protocols as well as on the mechanistic aspects of the action of current drug candidates.

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