3rd IAAASS

Innovative Approaches for Identification of Antiviral Agents Summer School

September 28th – October 3rd 2016,
Santa Margherita di Pula, Sardinia, Italy

Program & Abstract book

SARDEGNA RICERCHE

REGIONE AUTONOMA DELLA SARDEGNA

Fondazione Banco di Sardegna

International Antiviral Symposium Foundation

Dear Participant

Following the success of the previous editions, we have the pleasure to welcome you to the third “Innovative approaches for the identification of antiviral agents” summer school, with the patronage of Regione Autonoma della Sardegna, University of Cagliari, Sardinia Ricerche Research agency,European Society for Virology, Federation of European Microbiological Societies, Italian Society for Microbiology and Molecular Biologyand International Antiviral Symposium Foundation.

Over the recent years, drug discovery via high throughput screening (HTS) has moved beyond the boundaries of pharmaceutical companies and been successfully integrated into research programs of many academic institutes. These efforts have taken advantage of the availability of diverse libraries of small molecules, either as pure entities or natural product extracts. However, identification of candidate drugs benefits from an in-depth understanding of potential pitfalls of HTS, which can include (i), storage of compound libraries (ii), stability and cost of assay reagents (iii), robustness of the assay and (iv), access to bioinformatics to analyze the wealth of data that results from performing multiple assays on 250,000 – 500,000 compounds. Beyond these challenges, successful HTS is an iterative process requiring close co-operation with programs of structural biology, medicinal chemistry and clinical research.

With these issues in mind, the Innovative Approaches for Identification of Antiviral Agents Summer School (IAAASS) aims to provide an informal and interactive environment to review the application of HTS techniques to identification of novel and clinically-significant antiviral drugs. The Summer School is targeted to researchers at an early stage in their career, combining examples of drug discovery from internationally-recognized experts in the field with informal, small-group thematic discussion sessions.

The Organizing Committee welcomes you to the Sardegna Ricerche Research Park and to the Hotel Flamingo, Santa Margherita di Pula, located on the south tip of Sardiniaand looks forward to sharing with you their experience on current and future strategies for identifying novel antiviral agents targeted to clinically-significant diseases.

The summer school organizing committee

Organizing committee

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Consorzio per lo Studio dei Metaboliti Secondari

Università degli Studi di Cagliari
DIPARTIMENTO DI SCIENZE DELLA VITA E DELL'AMBIENTE

IAAASS program

IAAASS program

2016.09.28		
5.00 pm	Registration	
5:30 pm	Shuttle form Hotel Flamingo to the Research Park	
6.00 pm	**Remarks**	
	Enzo Tramontano	University of Cagliari, Committee Organizer
	Giorgio Palù	President of the European Society for Virology
	José Esté	President of the International Society for Antiviral Research
	Inaugural Lecture	
	Mike Bray *Antiviral Research editor*	Treatment of zoonotic viral infections: current status andfuture needs
8:00 pm	Shuttle form the Research Parkto Hotel Flamingo	
8:30 pm	Dinner	
2016.09.29		
8.40 am	Shuttle form Hotel Flamingo to the Research Park	
9.00 am	**Plenary lectures**	***Chairman: Cristina Parolin***
	Maurizio Botta *University of Siena (Italy)*	The First Molecule Interacting with a Host Protein for the Inhibition of Multiple Viruses
	Joel Schneider *National Cancer Institute (USA)*	Design of drug delivery vehicles that directly traverse the cell membrane
11.00-11.15 am	Coffee Break	
11.15 am	**Plenary lectures**	***Chairman: Mike Bray***
	Robert Jordan *GILEAD (USA)*	Discovery of a respiratory syncytial virus (RSV) fusion inhibitor GS-5806 and clinical proof of concept in a human RSV challenge study
	José Esté *Fundacia Irsicaixa, Badalona (Spain)*	Sensing of HIV-1 infection, a target for new therapeutic strategies
1.00 pm	**Lunch & Poster sessionI**	
2.30-4.00 pm	**Selected oral communications *Chairman: Elias Maccioni***	
2.30 pm	**Mertens Barbara** *Rega Institute KU Leuven, (Belgium)*	Cidofovir is active against human papillomavirus positive and negative tumor cells by causing DNA damage as one of its working mechanisms

2.45 pm	**Valentina N.Madia** *University La Sapienza(Italy)*	New insights into the Interaction between Pyrrolyl Diketoacids and HIV-1 Integrase Active Site
3.00 pm	**Pieter Vrijens** *Rega Institute KU Leuven, (Belgium)*	Role of receptor tyrosine kinases and associated gangliosides in the influenza virus replication cycle
3.15 pm	**Biesiada Marcin** *Polish Academy of Sciences(Poland)*	The specificity of GagΔp6, NC and MA interactions with 5'-UTR of HIV-2
3.30 pm	**Figueira Tiago N.** *Universidade de Lisboa (Portugal)*	Correlating self-assembling and lipid membrane interactions of cholesterolconjugated Measles Virus entry inhibitors with in vivo efficacy
3.45 pm	**Gloria Bua** *University of Bologna (Italy)*	Antiviral effect of cidofovir on Parvovirus B19 replication
4.00 pm	**Jenny Desantis** *University of Perugia (Italy)*	2-Catecholoxazinones as HIV-1 Ribonuclease H Inhibitors
4.30 pm	Shuttle form the Research Park to Hotel Flamingo	
6.00pm	Coffee break	
6.15-7.45 pm	**Discussion groups**	
8.30 pm	Dinner	

2016.09.30

8.40 am	Shuttle form Hotel Flamingo to the Research Park	
9.00 am	**Plenary lectures**	**Chairman*: José Esté***
	Giorgio Palù *University of Padova (Italy)*	Zika virus: from pathogenesis to disease control
	Stuart Le Grice *National Cancer Institute (USA)*	Re-purposing HIV RNase H inhibitors to target herpes virus nucleotidyl transferases
11.00-11.15 am	Coffee Break	
11.15 am	**Bio-tech session**	***Chairman: Stuart FJ Le Grice***
	Presentation of "Sardegna Ricerche" Research Park	
12.00 am	Round table: Working in a company after the PhD	
	Stuart FJ Le Grice (NCI)	
	Robert Jordan (GILEAD)	
	Vincenzo Summa (IRBM)	
1.30 pm	**Lunch & Bio-tech companies presentation desk**	
2.30-4.00 pm	**Selected oral communications**	***Chairman: Reuben Harris***
2.30 pm	**Quintana Verónica** *Universidad de Buenos Aires (Argentina)*	Characterization of the antiviral mode of action of anisomycin against dengue virus

2.45 pm	**Yipeng Ma** *KU Leuven, (Belgium)*	A novel early-stage inhibitor of Coxsackievirus B3 replication
3.00 pm	**Nicole Grandi** *University of Cagliari (Italy)*	HERV-W group characterization provides insights for potential innovative therapeutic targets for Multiple Sclerosis and other human diseases
3.15 pm	**Sara Pautasso** *University of Torino (Italy)*	The interferon-inducible DNA-sensor protein IFI16: a key player in the antiviral response
3.30 pm	**Francesco Saladini** *University of Siena (Italy)*	Application of cell based phenotypic assays for the evaluation of novel HIV-1 inhibitors targeting viral and host proteins
3.45 pm	**Iuni Trist** *University of Siena (Italy)*	Counteract influenza A by blocking PA-PB1 protein-protein interaction with the aid of molecular modelling
4.30 pm	Shuttle form the Research Park to Hotel Flamingo	
6.00 pm	Coffee break	
6.15-7.45 pm	**Discussion groups**	
8.30 pm	Dinner	
2016.10.01		
8.40 am	Shuttle form Hotel Flamingo to the Research Park	
9.00 am	**Plenary lectures**	***Chairman: Thomas Mertens***
	Graciela Andrei *KU Leuven (Belgium)*	Herpes virus drug resistance: where are we going? Challenges and opportunities
	Vincenzo Summa *IRBM (Italy)*	The discovery of Isentress, the first in class HIV-Integrase inhibitor
11.00-11.15 am	Coffee Break	
11.15 am	**Plenary lectures**	***Chairman: Stefano Alcaro***
	Reuben Harris *University of Minnesota (USA)*	DNA viruses and cancer mutagenesis
	Maria Josè Camarasa *IQM-CSIC (Spain)*	A medicinal chemistry approach for the discovery of inhibitors HBV and (re)emerging virus replication
1.00 pm	Lunch	
2.30 pm	Shuttle form the Research Park to Hotel Flamingo	
	Free afternoon	
8.30 pm	Dinner	
2016.10.02		
8.40 am	Shuttle form Hotel Flamingo to the Research Park	
9.00 am	**Plenary lectures**	***Chairman: Robert Jordan***
	Thomas Mertens *University of Ulm (Germany)*	Reinforcement of natural immunity as an option for antiviral therapy

	Stefano Alcaro *"Magna Graecia" University (Italy)*	Computational tools for multi-targeting novel bioactive compounds
11.00-11.15 am	Coffee Break	
11.15 am	**Plenary lectures**	***Chairman: Graciela Andrei***
	Katarzyna Purzycka *Polish Academy of Sciences (Poland)*	Structural studies of retroviral RNAs
	Anna Papa *University of Thessaloniki (Greece)*	Emerging viral diseases in the Mediterranean region
1.00 pm	**Lunch & Poster session II**	
2.30-4.30 pm	**Selected oral communications**	***Chairman: Katarzyna Purzycka***
2.30 pm	**Joanna Sztuba-Solinska** *CCR NCI,Frederick, MD (USA)*	A small Stem-Loop Structure of the Ebola Virus Trailer interacts with HSPA8 and is essential for Ebola Virus Replication
2.45 pm	**Liu Ching-Hsuan** *Taipei Medical University(Taiwan)*	Highly Bioavailable Silibinin Nanoparticles Inhibit Hepatitis C Virus
3.00 pm	**Angela Corona** *University of Cagliari (Italy)*	Exploring the hydrazoindolin-2-one based scaffold to develop Ribonuclease H/DNA polymerase HIV-1 RT dual inhibitors
3.15 pm	**Carla Usai** *Universidad de Navarra*	Characterisation of a mouse model for the study of HDV chronic infection
3.30 pm	**Maria Elena Terlizzi** *University of Torino (Italy)*	The A-type Proanthocyanidins (PACs-A) of a novel cranberry extract inhibit Herpes Simplex Type 1 and Type 2 infections
3.45 pm	**Scano Alessandra** *University of Cagliari (Italy)*	Future Perspectives in Antiviral drug delivery
4.15 pm	Shuttle form the Research Park to Hotel Flamingo	
6.00pm	Coffee break	
6.15-7.45 pm	**Discussion groups**	
8.30 pm	Dinner	
2016.10.03		
	Breakfast and leaving	

Plenary lectures abstracts

Treatment of zoonotic viral infections: current status and future needs

Mike Bray

Editor-in-Chief, Antiviral Research Chevy Chase, MD, USA

Researchers have had great success over the past three decades developing antiviral therapies for a number of human viral infections, beginning with the herpesviruses, continuing with HIV and hepatitis C, and with the prospect of curative treatment of hepatitis B “on the horizon.” However, achieving approved therapies forviral infections transmittedfrom animals has been much more challenging. I will review a range of important viral zoonoses, including a brief summary of their epidemiology and clinical features, and discuss steps that will be needed to bring effective therapies from the laboratory bench to the patient.

NOTES

The first moecule interacting with a host protein for the inibition of multiple viruses

Maurizio Botta

Dipartimento Biotecnologie, Chimica e Farmacia, Università degli Studi di Siena, Via A. Moro 2, I-53100 Siena, Italy.

The cellular helicase DEAD-box 3 (DDX3) is known to be an essential host factor for major human viral pathogens such as HIV-1 and Hepatitis C viruses as well as for the replication of viral agents responsible for orphan diseases such as Dengue virus (DENV), West-Nile virus (WNV), Human T-cell leukemia Virus (HTLV)-1 and Japanese Encephalitis Virus (JEV). No specific and effective pharmacological treatment is currently available for these latter pathogens, despite being an increasing threat to EU citizens that may eventually lead to sustained epidemics in Europe. Additionally, all compounds that are currently approved for the treatment of other viral infections target viral proteins. Targeting a unique viral function has an important the Achilles' heel: viral resistance to the drugs, an important threat to the efficacy of current therapy. Conversely, the alternative strategy, targeting a cellular factor that is required for viral replication, should help to overcome this problem. Theoretically, a drug targeting a cellular factor could also inhibit all viruses that are dependent on the same host factor. Recently, it has been revealed that the cellular ATPase/RNA helicase X-linked DEAD-box polypeptide 3 (DDX3) is an essential host factor for the replication of several viruses.1 Accordingly, our research group is working in targeting both the ATPase and RNA binding regions of DDX3.2-6 Most of the synthesized derivatives were able to inhibit the DDX3 helicase activity at submicromolar concentration. Furthermore, these compounds showed anti-HCV and anti-HIV activity in cells, as well as a good inhibitory activity against JEV, DENV and WNV infections. Our results clearly demonstrated that DDX3 inhibitors could be exploited in order to treat HIV/HCV co-infections, emerging infectious diseases such as Dengue and West Nile and HIV-1 patients carrying drug resistant strains. Each of these three medical conditions currently represents a major challenge for clinical treatment.

References:
[1] Schroder M., 2010, Biochem. Pharmacol., 79, 297
[2] Maga G. et al 2008, J. Med. Chem., 51, 6635
[3] Maga G. et al 2011, ChemMedChem, 6, 1371
[4] Garbelli A. et al., Curr. Med. Chem., 18, 2094
[5] Radi M. et al. 2012, Bioorg. Med. Chem. Lett., 22, 2094
[6] Fazi R. et al. 2015, J. Chem Inf Model., 11, 2443

NOTES

Design of Drug Delivery Vehicles that Directly Traverse the Cell Membrane

Joel Schneider

Chemical Biology Laboratory, National Cancer Institute, National Institutes of Health

Many lead molecules identified in drug discovery campaigns are eliminated from consideration due to poor solubility and low cell permeability. These orphaned molecules could have clinical value if solubilized and delivered properly. Developing methods to deliver small molecules to cells remains a daunting challenge. We designed a peptide, named SVS-1, that preferentially folds at the surface of tumor cells, adopting a β-hairpin conformation that rapidly enters cells. SVS-1 is stable in serum and small molecules attached to the peptide are effectively delivered to cancer cells via mechanisms involving physical translocation and, to a lesser extent, clathrin-dependent endocytosis. Many cell penetrating peptides (CCPs), including SVS-1, fold at the surface of cells, adopting α- or β-structure that enable their intracellular transport. However, the very same structural folds that facilitate cellular entry can also elicit potent membrane-lytic activity limiting their use in delivery applications. Further, one distinct CPP can enter a cell via a myriad of mechanisms, many of which lead to endosomal entrapment. Using SVS-1 as a structural template, we designed a second generation CCP named CLIP6 that is intrinsically disordered and exclusively employs non-endosomal mechanisms to cross the lipid membrane of cells. The presence of a single anionic glutamate residue is responsible for maintaining the peptide's disordered bioactive state, defines its mechanism of cellular entry and is central to its biocompatible nature. CLIP6 can deliver membrane impermeable cargo directly to the cytoplasm of cells, suggesting its broad utility in the delivery of drug candidates currently limited by poor cell permeability and endosomal degradation.

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Discovery of Presatovir (GS-5806), an Orally Bioavailable Respiratory Syncytial Virus (RSV) Fusion Inhibitor

Robert Jordan

Gilead Sciences, Inc. Foster City, CA 94404

RSV is the leading cause of bronchiolitis and pneumonia in young children and is associated with severe respiratory disease in the elderly and immunocompromised individuals. Virazole® (ribavirin), delivered as an aerosol, has been used to treat RSV but has shown equivocal efficacy. Thus, new antivirals are needed to treat RSV infections.

A high throughput screen of over 500,000 compounds was conducted to identify inhibitors of RSV replication. Hit compounds were organized based on their chemical structures and ranked according to antiviral activity and chemical tractability. Selected representatives from top compound classes were chemically optimized to improve antiviral potency and drug-like properties. Lead compounds were evaluated for further development using in vitro absorption, distribution, metabolism and excretion (ADME) assays and in vivo measuring oral bioavailability, drug distribution to the lungs and pharmacokinetics. This optimization campaign lead to the identification of Presatovir which demonstrated potent (EC50 = 0.43nM) antiviral activity and favorable drug-like properties.

In vitro cell-based and biochemical assays demonstrated that Presatovir targets the RSV F protein and inhibits F protein-mediated fusion of the virus envelope with the host cell membrane. The pharmacokinetics and toxicity of Presatovir was evaluated in rats, dogs, and non human primates. The antiviral efficacy of Presatovir or related analogs was evaluated in cotton rats, calves and a human challenge model. In the human challenge model Presatovir was found to be safe, well tolerated, and significantly reduced disease symptoms and viral load in nasal secretions. Presatovir is in Phase 2 development for treatment of RSV infection.

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Sensing of HIV-1 infection, a target for new therapeutic strategies

José Esté

AIDS Research Institute – IrsiCaixa, Universitat Autónoma de Barcelona, Badalona, Spain.

The result of any viral infection depends largely on innate and adaptive immune responses that accompany it. The innate response against HIV-1 is an essential element in the establishment of the primary infection since dendritic cells or resident tissue macrophages are possibly responsible for the transmission of virus to CD4+ T cells or the transport of viral particles to lymphatic tissue. We are beginning to understand the mechanisms of virus restriction in monocytes and cells of the myeloid lineage that represent the first line of defense against HIV-1 infection. We have elucidated the pathway associated to activation/inactivation of HIV-1 restriction in primary macrophages and how this is directly linked to cell cycle control, cell proliferation and the activation of an interferon-mediated antiviral response. Additionally, the interplay between innate immune sensing and clearance of intracellular DNA has important implications for the understanding of responses to infectious retroviruses.

Intracellular pathways leading to sensing of foreign or aberrant genomic materials (DNA or RNA) and triggering of an interferon response are being studied as potential targets for drug development. The regulation an interferon-mediated immune response may lead to new strategies to reduce chronic activation and T cell depletion associated to HIV infection. Additionally, agents targeting specific components of the recognition of viral genomic material and enhancement of innate defenses may serve to effectively combat chronic infection and reduce the latent HIV-1 reservoir.

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Zika virus: from pathogenesis to disease control

Giorgio Palù

University of Padova. Italy

Zika virus is a mosquito-borne flavivirus discovered in Uganda in 1947. The virus has emerged in the recent years and spread in the Pacific Area and the Americas, where it has caused large human outbreaks. Factors involved in virus emergence are still unknown, but probably included the introduction in naïve environments characterized by the presence of high densities of competent Aedes spp. mosquitoes and susceptible human hosts in urban areas. Unique features of Zika virus infection are sexual and transplacental transmission and associated neurological morbidities, i.e. Guillain-Barré syndrome and foetal microcephaly. Diagnosis relies on the detection of viral nucleic acids in biological samples, while detection of a specific antibody response may be inconclusive because of the broad cross-reactivity of antibodies among flaviviruses. Experimental studies have clarified some mechanisms of Zika virus pathogenesis and have identified potential targets for antiviral drugs. In animal models, the virus can infect and efficiently replicate in the placenta and in the brain and induces foetal demise or neural damage, recapitulating human diseases. These animal models have been used to evaluate candidate vaccines and promising results have been achieved. Personal experience on the investigation of Zika virus infection in humans and neuropathogenesis will be presented, in the context of data from the literature.

NOTES

Re-purposing HIV RNase H inhibitors to target herpes virus nucleotidyltransferases.

Stuart F.J. Le Grice

Basic Research Laboratory.National Cancer Institute, Frederick, MD, USA

Herpes simplex viruses 1 and 2 (HSV-1, HSV-2) areclosely-related enveloped a-herpesviruses with large, double-stranded DNA genomes encoding ~80 proteins. Etiologically, HSV-1 is associated with gingivostomatitis, herpetic stromal keratinitis and anogenital lesions, while HSV-2, the primary agent of ulcerative anogenital lesions, and which infects ~20% of individuals in the US, has been documented to increase the frequency of human immunodeficiency virus (HIV) acquisition. DNA polymerase has been the primary HSV antiviral target, exemplified by the deoxyguanosine analog acyclovir (ACV), the drug of choice for almost 40 years. However, an increased incidence of ACV-refractory HSV keratitis, a leading cause of corneal morbidity in industrialized countries has been reported. Recent reports from immunocompetent patients with recurrent herpetic keratitis receiving ACV treatment indicates that ACV-resistance may be more common that previously recognized in this particular population. The emergence of viral resistance to this class of compounds thus suggests a need for alternative therapies with increased efficacy and improved pharmacokinetics. Based on the effectiveness of combination antiretroviral therapy in treating HIV infection, a cocktail of agents for HSV showing acceptable pharmacodynamic profiles and distinct molecular targets would likewise be expected to significantly reduce viral load and delay acquisition of drug resistance.

Inhibition of wild type and ACV-resistant HSV-1 and HSV-2 replication by several natural product troponoids, including manicol and β-thujaplicinol was recently reported. Although the viral target remains to be established, α-hydroxytropolones potently inhibitribonuclease H (RNase H) activity of HIV-1 reverse transcriptase by sequestering the catalytically-critical divalent metal at the active site. Thus, a structurally-related HSV enzyme would seem a likely HSV target. One candidate, pUL15, is a component of the terminasemolecular motor complex responsible for "pumping" viral DNA into the capsid. The pUL15 C-terminal nuclease domain (pUL15C) uses a 2-metal ion-mediated catalytic mechanism to cleave concatemericviral DNA, and a recently-reported crystal structure has highlighted the presence of an RNase H-like fold common to nucleotidyltransferases (NTases). However, the previous methodology for assaying pUL15C activity required cleavage of supercoiled DNA and agarose gel electrophoresis, which is time consuming, challenging to quantify, and impractical for high throughput screening (HTS). We have used several short, closely-related oligonucleotide duplexes (~21 bp) to interrogate pUL15C nuclease activity, showing that relatively minor changes can affect both the kinetics and specificity of cleavage. Features of this assay were incorporated into a simple and rapid dual-probe fluorescence assay for high throughput screening.By combining differential scanning fluorimetry with the dual-probe fluorescence assay, two facile screening tools for an HSV nuclease that can be extended to orthologs of other herpesviruses will be discussed.

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Experience of a translational research platform for the evaluation of drug-resistance among Herpesviruses

Graciela Andrei

KU Leuven (Belgium)

Drug-resistance in herpesviruses is virtually not observed in immunocompetent individuals but it is a well-recognized problem in immunocompromised patients. Therefore, in 2009 a Reference and Service Center, RegaVir [Research Group for Antiviral Resistance, (www.regavir.org)], for the diagnosis and typing of drug-resistant herpesviruses was established in Belgium.

Phenotyping (drug-susceptibility profile) and/or genotyping {PCR amplification of viral genes involved in drug-resistance [UL97 protein kinase and DNA polymerase (DNA pol) for cytomegalovirus (CMV); thymidine kinase and DNA pol for herpes simplex virus (HSV) and varicella-zoster virus (VZV), and U69 protein kinase and DNA pol for human herpesvirus 6 (HHV-6], followed by DNA sequencing} are used to diagnose drug-resistance among herpesviruses according to the virus and the type of sample. Today, in the context of the RegaVir platform, we have analyzed 1050 clinical samples recovered from patients that were refractory to antiviral therapy. Our data show: a) the usefulness of rapid genotyping and/or phenotyping for the adjustment of antiviral therapy, b) a considerable number of isolates bearing mutations linked to drug-resistance among the samples that proved positive for virus isolation and/or PCR amplification, c) the identification of unknown genetic polymorphisms and of novel mutations linked to drug-resistance, d) a higher risk for developing drug-resistance infections in the central nervous system, e) emergence of multiple drug-resistance conferred by infection with multiple viral strains, f) compartmentalization of drug-resistant herpesviruses and, g) the need to extend the present platform to other viruses such as adenovirus and polyomavirus

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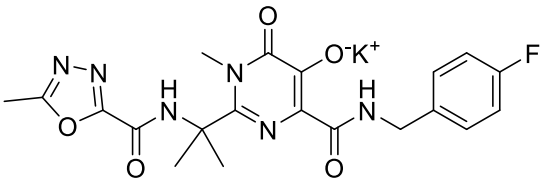
The discovery of Isentress™, the first in class HIV Integrase inhibitor

Summa Vincenzo

Vincenzo Summa PhD, Senior Executive Director,IRBM Science Park. Pomezia (RM) ITALY

The presentation will describe the discovery history of **Isentress™ (Raltegravir), starting from the initial hit trough all phases of the drug development that led to the final** FDA **approval as** the first in class HIV-integrase inhibitor approved for the treatment of HIV infection. In particular, the presentation is a journey through all aspects of the drug discovery process and highlights the strategies applied to overcome the issues encountered during a very challenging drug discovery project. We will review the structure activity relationships, the synthetic strategies, the pharmacokinetic and metabolic profile, the ancillary pharmacology results on key compounds and finally the clinical trials that allowed the approval of MK-0518 as new therapeutic agent for the treatment of the patients affected by the HIV infection.

The discovery of Isentress™ is a very good example of interdisciplinary team work and creative medicinal chemistry strategy.



Isentress™, Raltegravir

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Targeting Virus and Tumor Evolvability

Reuben S. Harris

Howard Hughes Medical Institute, University of Minnesota, Minneapolis, Minnesota, USA

Cancers often display incredible genetic heterogeneity characterized by hundreds of gross chromosomal aberrations and tens of thousands of somatic mutations. Tumor evolution is thought to be ongoing and the sum of all exogenous and endogenous mutagenic processes. Many viruses evolve at much higher rates, and virus evolution is thought to be similarly complex with both viral and cellular processes contributing to overall levels of genetic variation. Historically, tumor and virus evolution are thought of as largely independent processes. However, many studies have converged recently on the antiviral enzyme APOBEC3B as a major source of virus and tumor mutagenesis and evolution. This lecture will highlight recent progress in this area, review data showing how small DNA tumor viruses (HPV and PyV) specifically upregulate APOBEC3B, and discuss the therapeutic feasibility of starving viruses and tumor cells of mutational fuel.

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A Medicinal Chemistry Approach for the discovery of Inhibitors of HBV and (re)emerging Virus Replication

María-José Camarasa

Instituto de Química Médica (IQM-CSIC), 28006 Madrid, Spainmj.camarasa@iqm.csic.es

Infectious diseases have been a cause of concern for humans over millennia and nowadays the expansion of an outbreak in one part of the world is often unpredictable as it is facilitated by the ease of world travel and the global exchange of goods. Two major categories of infections can be defined, namely, newly emerging diseases that are recognized in the human host for the first time and reemerging diseases that historically have infected humans but continue to appear in new locations or in drug-resistant forms, or reappear after apparent control or elimination, often accompanied by significant changes in pathogenicity (HIV infection is one example of emerging viral infections). These diseases have a profound global economic and social impact in relation to illness-related deaths and also in the interference with normal life activities. Due to the capacity of RNA viruses for rapid mutation these viruses are particularly prone to adaptation to environmental changes. In addition, the mutability of RNA viruses is one of the reasons that explain the lack of efficacy of vaccination against these pathogens.

For a number of years our research group has been deeply involved in the design and discovery of new antivirals, an area in which there are still largely unmet medical needs affecting humans, particularly highly prevalent pathologies such as AIDS, and more recently in HVB and (re)emerging pathogens that seriously compromise human health. In this presentation our efforts to design inhibitors of the replication of two different (re)emerging RNA virus, namely, chikungunya and enterovirus 71 and two highly prevalent emerging viruses as HIV and HBV will be discussed.

NOTES

Reinforcement of natural immunity as an option for antiviral therapy

Thomas Mertens

Enormous progress was possible in antiviral therapy during the last decades. Nevertheless, a number of serious problems has also become apparent during extensive clinical application of many antiviral substances such as drug toxicity and selection of drug resistant mutants. On the other hand, it has often been observed that durable success of antiviral therapy is dependent on residual immune functions of the patient. For many reasons immune therapy is a very attractive treatment option. In clinical practice passive transfer of virus specific T-cells is adopted for the control of life threatening EBV-, CMV- and other infections in severely immunocompromised patients. Concerning many aspects the role and mechanisms of innate immunity and effector cells for the control of human viral infections is far from being clear. Understanding of innate immune functions is essential for identification of potential targets for modulation but needs novel experimental approaches.

NKG2Chi CD57hi NK cells can be identified as HCMV-induced NK cells, which are highly responsive to HCMV-infected autologous macrophages only in the presence of HCMV-specific antibodies. So the activity of pathogen-induced innate immune cells can be enhanced by adaptive humoral immunity.

On the other hand, peripheral blood NK cells (PBNKs) from HCMV seropositive donors showed an enhanced activity towards HCMV infected autologous macrophages. However, this memory-like response was abolished when purified NK cells were applied as effectors and the activity was shown to be dependent on the IL-2 secretion of CD4+ T cells when re-exposed to the virus. So NK cell mediated innate immunity can be enhanced by a preexisting T cell antiviral immunity with potential implications for HCMV treatment.

Experimentally NK cells can efficiently control HCMV transmission in different cell types, engaging different mechanisms to control the HCMV transmission both via soluble factors and by cell contact. NK cell produced interferon gamma (IFN-γ) suppresses HCMV production and induces resistance of bystander cells to HCMV infection. Interestingly a dynamic expansion of NKG2C+ NK cells even in a RAG-2 deficient patient was observed post infection. This reveals the antiviral activity of human RAGs-/ DCLRE1C--NK cells. Our findings indicate a clinical relevance of NK cells in HCMV infection and highlight the need to consider potential therapeutic strategies based on the manipulation of NK cells.

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Computational tools for the development of multi-targeting bioactive agents

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Recently the traditional medicinal chemistry paradigm of "one target, one drug" has been reformulated accepting the philosophy and the potential advantages of active compounds recognising at the same time two or more biological targets.1 This new paradigm is extremely interesting at both academic and industrial levels. Actually, the term "repurposing" is one of the modern strategy for revaluating unused or underdeveloped compounds, especially within research groups of Pharma companies.

On the other hand the multi-targeting issue represents a significant complication for the drug discovery procedures and protocols. How to investigate in a fast manner potential multi-targeting profiles of old/novel drugs? How to deal and balance within their multiple activity profiles? These are only first two questions for medicinal chemists to deal with. Computational methods and approaches, widely used at academic and industrial level in Italy,2 can give a consistent support to answer to these issues. In this communication some of them will be briefly analysed and linked to the multi-targeting drug discovery problem.

Moreover, the recently approved COST Action MuTaLig (Multi-target paradigm for innovative ligand identification in the drug discovery process) started to join highly-qualified research teams working in disciplines around multi-target issue in drug discovery. Started as 5 co-proposing European research teams the joined COST parties are now 30 countries. The COST Action will be active for 4 years up to 2020. Additional details about the MuTaLig COST CA15135 Action with the support of the EU Framework program Horizon 2020 are available on line.3

References

[1] Medina-Franco J. L., Giulianotti M. A., Welmaker G. S., Houghten R. A. *Drug Discovery Today*, 2013, *18*, 495-501.

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[3] www.cost.eu/COST_Actions/ca/CA15135 and www.mutalig.eu

NOTES

Structural studies of retroviral RNAs

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RNAs adopt specific structures to perform their activities. RNA structure is influenced by primary sequence and cellular environment. The ability of RNA strands to fold back on themselves to form stable tertiary architectures is fundamental to RNA function. Specific proteins might interact with RNA that stabilize and/or protect its native structure.

An interesting feature of retroelements, such as HIV-2 retroviruses and related Ty1 retrotransposons, is their genomic RNA (gRNA) that plays dual role in replication. gRNA serves as a template for translation as well as the retroelement's genome that is packaged into particles. Retroelements' gRNAs contain internal structures fundamental to propagation. Prominent among these motifs are cis-acting sequences required for gRNA dimerization, packaging and priming of reverse transcription. Recent advances in recognizing the ways RNAs control viral pathogenesis make it certain that RNA structures will become increasingly important targets for therapeutic intervention.

Advancements in RNA secondary structure probing methods and prediction algorithms allow to generate reasonable models of the consensus secondary structure. Determination of RNA tertiary structure still remains a significant challenge due to RNAs dynamic nature. However, new computational strategies are emerging for RNA 3D structure predictions.

Using experimental and computational approaches we are developing secondary and tertiary structure models of retroelements RNAs. Combining structural studies with the molecular and genetic methods allows us to study structure/function relationships of HIV-2 and Ty1 retroelements' gRNAs.

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Emerging viral deseases in the Mediterranean region

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Most of the emerging viral diseases in the Mediterranean region are caused by arboviruses (viruses transmitted by arthropods, such as ticks, mosquitoes, sandflies) which constitute a significant Public Health threat. Among **tick-borne viruses**, Crimean-Congo hemorrhagic fever virus (CCHFV) emerged in 2002 in Turkey with thousands of cases through 2016. The first CCHF case in Greece was reported in 2008, while the virus emerged in Spain in September 2016. The first tick-borne encephalitis cases have been reported recently in Bulgaria and Greece. Regarding **mosquito-borne viruses**, West Nile virus (WNV) caused large outbreaks in the area; it is of interest that the outbreaks in Balkans were caused by WNV lineage 2, while the outbreaks in Turkey and Israel were caused by WNV lineage 1. Both lineages circulate in Italy, together with the Volgograd strain (known to circulate in Eastern Europe) which has been detected in 2014 in mosquitoes. A few autochthonous cases of Dengue and Chikungunya virus infections have been reported in Southern France and Croatia. Numerous imported cases of Zika virus infections have been reported in Europe, with a risk for authochthonous cases in places where competent mosquitoes are present. Several novel **sandfly-transmitted phleboviruses** have been indentified recently in the Mediterranean countries, some of them associated with disease in humans. Many biotic and abiotic factors play a role in the emergence of arboviral diseases. Awareness of medical staff, reporting the unusual, and surveillance are needed. There is an urgent need for drug design, especially for the viruses which cause severe human disease and/or those associated with congenital infections.

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Short talks abstracts

The specificity of GagΔp6, NC and MA interactions with 5'-UTR of HIV-2

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The Gag polyprotein is a multifunctional regulator of retroviral replication and major structural component of immature virions. HIV Gag comprises matrix (MA), capsid (CA), nucleocapsid (NC) domains, two spacer regions and p6 domain. This polyprotein is cleaved into freestanding MA, CA and NC proteins during virion maturation. The nucleic acid chaperone (NAC) activity is considered necessary to retroviral Gag functions, but so far, NAC activity has only been confirmed for HIV-1 and RSV Gag polyproteins.
We found that HIV-2 Gag lacking p6 domain is a robust nucleic acid chaperone [1].Moreover, we present the evidence that HIV-2 MA displays NAC activity and propose that MA domain may enhance the activity of HIV-2 GagΔp6. HIV-1 MA does not display NAC activity. Comparison of HIV-1 and HIV-2 MA proteins suggests reasons for observed differences.
As a nucleic acid chaperone, Gag binds NA non-specifically, but is also engaged in highly specific recognition of *cis*-acting dimerization and packaging (Ψ) signals within the 5′UTR of the viral genomic RNA. Therefore, using hydroxyl radical footprinting we characterized HIV-2 Gag, NC, and MA interactions with HIV-2 5'UTR. The HIV-2 NC and GagΔp6 show strong binding to the packaging signal (Ψ) of HIV-2 RNA and preference for the purine-rich sequences. MA protein binds mainly to G residues without favouring Ψ RNA. Our data on HIV-2 indicate that the role of the MA domain in the NAC activity of Gag differs not only between, but also within, retroviral genera. Studies of Gag/RNA interactions may help drug discovery targeted to HIV.

1 Pachulska-Wieczorek et al. *Retrovirology***2016**, 13, 18.

Antiviral effect of cidofovir on Parvovirus B19 replication

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The acyclic nucleoside phosphonate cidofovir (CDV) has broadly activity against dsDNA viruses. The present study was aimed to investigate an extension of its activity to parvovirus B19 (B19V), a ssDNA virus lacking of a vaccine and of a causative therapy. [1]
Two model systems were used to assess the activity of CDV on B19V replication: UT7/EpoS1 cell line and ex vivo-expanded EPCs (erythroid progenitor cells).[2] Different multiplicity of infections (10^0-10^4 genomes/cell) and CDV concentrations (0.1-500 μM) were tested. B19V DNA was analysed at 2 hpi and 72 hpi in UT7/EpoS1 cells and 24 hpi in EPCs. Moreover, cellular assays were performed to measure effects of CDV on cell proliferation and viability. Results demonstrated that B19V replication on UT7/EpoS1 cells is potently inhibited by CDV at all the tested moi and in a dose-dependent manner, yielding to a EC50 of 7.45-41.27 μM. On EPCs, CDV showed a lower effect provoking significant reduction of B19V replication (68.2-92.8 %) when used at 500 μM. With regard to the host cells, CDV possessed neither cytostatic nor cytotoxic effects, suggesting a selective activity on the virus. Herein, different experimental approaches were carried out to explore a possible enhancement of CDV activity in EPCs.[3] Viral inhibition was enhanced in infected EPCs extendedly exposed to 500 μM CDV and in serially infected EPCs with passage of the virus progeny, constantly under drug exposure. In addition, 92% of inhibition was reached in EPCs treated with 500 μM CDV 24 h before infection.
The work is the first evidence of inhibitory activity of an antiviral compound against B19V and is promising for the development of antiviral drugs against B19V.

1Bonvicini et al. *Antiviral Res***2015**, 113, 11-18.
2Bua et al. *PLoS ONE***2016**, 11, e0148547.
3 Bonvicini et al. *Virus Res***2016**, 220, 47-51.

Exploring the hydrazoindolin-2-one based scaffold to develop Ribonuclease H/DNA polymerase HIV-1 RT dual inhibitors.

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The DNA polymerase and ribonuclease H (RNase H) activities of human immunodeficiency virus type 1 (HIV-1) are needed for the replication of the viral genome and are validated drug targets. However, there are no approved drugs inhibiting RNase H and the efficiency of DNA polymerase inhibitors can be diminished by the presence of drug resistance mutations. In this context, drugs inhibiting both activities could represent a significant advance towards better anti-HIV therapies.
Here we report structural studies of optimization of a new isatine-based scaffold, recently identified as good start point for dual RT inhibitors active in the low micromolar range and active against HIV-1 variants naturally selected as resistant to currently approved allosteric Non nucleoside RT inhibitors. Hydrazoindolin-2-one derivatives act by binding to two different RT pockets: one in the RNase H domain, responsible for inhibition of the RNase H function , one in the DNA polymerase domain, affecting the RT-associated DNA polymerase function. The (Z)-4-(2-(2-(2-oxoindolin-3-ylidene)hydrazinyl)thiazol-4-yl)benzonitrile structure has been used as hit compound to generate a small library of 58 new compounds with the aim to improve the potency of inhibition and to gain antiviral activity, inserting various functional groups. Among the synthesized compounds, 32 were active in the low/sub micromolar range, with a good improvement in potency respect to previously reported analogs. Structure activity relationships allowed us to better define the differences in molecular space between the two pockets, gaining new insights on modulating the inhibitory activity against the two functions.

2-Catecholoxazinones as HIV-1 Ribonuclease H Inhibitors

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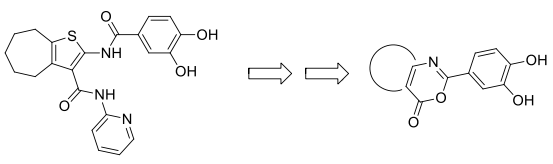
The inability of current antiviral drug regimen to eradicate HIV-1 together with the rapid emergence of multidrug-resistant viral strains clearly underline the need to search for more effective anti-HIV agents, possibly endowed with innovative mechanism of actions. In this contest, owing to its pivotal role in HIV genome replication, the reverse transcriptase-associated ribonuclease H (RNase H) function has proven to be an appealing target for the development of future combination antiretrovirals. While no drug has reached clinical trials yet, during the last two decades different research groups focused on the identification of RNase H inhibitors, mainly targeting its active site.[1]By screening an *in-house* series of cycloheptathiophene-3-carboxamide derivatives, previously found to be active as anti-Flu agents,[2] we have identified compounds endowed with inhibitory activity against RNase H.A successive structural optimization study led to identify a catechol derivative (compd **1**, Figure **1**) with IC_{50} value on the RNase H activity in the nanomolar range and endowed with a selective allosteric mechanism of inhibition.[3]With the aim of investigating this new class of RNase H inhibitors, a series of rigid analogues, i.e. thieno[2,3-*d*][1,3]oxazin-4-one-based compounds, has been now synthesized confirming the catechol moiety as the most suitable to impart anti-RNase H activity. Additional catechol-based derivatives have been developed by modifying the oxazin-4-one scaffold, leading to the identification of many derivatives endowed with nanomolar inhibitory activity.In this work, the design, synthesis, biological evaluation and mechanistic studies of a large series of catechol derivatives, will be presented.



Compd **1**

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2 Massari, S. et al. *J. Med. Chem.* **2013;** 56, 10118-31.
3 Corona, A.; Desantis, J.; et al. *ChemMedChem.***2016.**

Correlating self-assembling and lipid membrane interactions of cholesterolconjugatedMeasles Virus entry inhibitors with in vivo efficacy

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Measles Virus (MV) infection remains one of the leading causes of child mortality worldwide and isexperiencing considerable reemergence due to poor vaccine coverage and import of the virus fromendemic areas1. Moreover, there are no MV-specific treatment options available in clinical practice.Recently developed MV entry inhibitor peptides targeting the envelope Fusion protein have shownpromising results in an in vitro model of infection2. These peptides were chemically conjugated witheither cholesterol (Chol) or tocopherol (Toc) to promote concentration near membrane-proximal targetsites and dimerized to improve their avidity towards the F protein pre-fusion conformation. In vivo datasuggest that membrane integration, enhanced biodistribution and longer half-life are important for theinhibitory action of these conjugated peptides. Through biophysical approaches, we show thatchemical conjugation with either Chol or Toc leads to peptide nanoparticle self-assembling andimproved lipid membrane interactions. Although stable in solution, nanoparticles (~100 nm) allowefficient peptide insertion into membranes with increased affinity when compared to non-conjugatedcontrols. Lipid compositions mimicking either the cell membrane or viral envelope retain peptideconjugates efficiently, which dissociate at very slow rates over time.We propose that peptide self-assembly and lipid membrane interactions are responsible for thebiodistribution and bioavailability profiles observed in vivo. Additionally, improved retention in viral andcell membranes may help explain their improved inhibitory efficacy.

1 Abad, C.L.& Safdar, N. *Curr Infect Dis Rep***2015**, 17, 51.
2 Welsch, J.C. et al. *J Virol***2013**, 87, 13785–13794.

HERV-W group characterization provides insights for potential innovative therapeutic targets for Multiple Sclerosis and other human diseases.

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Human Endogenous Retroviruses (HERVs) are remnants of ancient viral integrations, constituting the8% of our genome. During their persistence, HERVs accumulated mutations that compromised theircoding capacity. A prominent exception is the HERV-W group member in locus 7q21.2, able toproduce a functional Env protein (Syncytin-1) coopted for placental development. Moreover, the wholegroup is of particular interest due to many studies connecting it to human diseases, such asSchizophrenia, Bipolar Disorder, Osteoarthritis, Lymphoma and especially Multiple Sclerosis (MS).These associations are based on the generic presence of HERV-W transcripts/antigens in patientssamples and on the evidence that HERV-W Env proteins are able to induce autoimmunity and MS-likeencephalitis. A major obstacle to the proper definition of HERV-W pathogenic role was the lack of adetailed and updated knowledge of its single members, essential to definitively assess any specificetiological determinant.In the present work we characterized in detail 213 HERV-W elements, using an innovativebioinformatics approach on an updated version of the human genome. These elements have beencharacterized in terms of structure, phylogeny and genomic context, providing an exhaustive picture ofthe group. Our dataset represents the most complete HERV-W library up to date and, importantly,allows to unambiguously identify the uniqueness of each single member. This make it particularlyvaluable to connect MS expression profiles to their genetic determinants, as already done for MS, inorder to identify specific biomarkers and innovative pharmacological targets in the context of theHERV-W connected diseases.

Anovel early-stage inhibitor of Coxsackievirus B3 replication.

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Coxsackievirus B3(CVB3) is a non-enveloped, positive-sense RNA virus that belongs to the genus enterovirus within the family of the Picornaviridae. The virus is among others associated with viral cardiomyopathies in infants and toddlers. In a cell-based antiviral screen, a small molecule(here denominated B14) was identified as inhibitor of in vitro CVB3 replication with an EC50of 9.1±1.5 μM. The activity of twenty-three analogues was evaluated and a more potent analoguewas found with an EC50 of2.6±0.5 μM. The activity of B14 was further confirmed by measuring the RNA virus yield (EC50 of 5.6±0.7 μM). A time-of-drug addition assay indicated that B14 exertsits activity at the early stage of virus replication. Rupintrivir, a broad-spectrum enterovirus 3C protease inhibitor was used as late-stage inhibitor control. Next, by using a clonal resistance selection protocol, six compound-resistant viruseswere isolated. Phenotypic characterization of these resistant isolates revealed >100-fold resistance to B14. Seven mutations in the gene of capsid protein VP1 were detected. Single mutants VP1_Y75C, VP1_A88V, VP1_A98V, VP1_D133N and VP1_R219K showed 15-, 2-, 4- 17- and 76-fold resistance to B14, respectively, while single mutants VP1_I28M and VP1_T36M were not resistant. Thermostability studies revealed that B14 could protect CVB3WT from heat inactivation. The compound protected as well as the five resistant mutants against high temperatures. Furthermore, the plaque size of VP1_A88V, VP1_D133N and VP1_R219K was smaller than WT and these mutants werealso more heat-sensitive than WT in the absence of the compound. This finding suggests that these three mutations may increase virion capsid flexibility and compensate the stabilizing effect of B14. Molecular modelling of B14 suggests that the compound may binds to a small cavity surrounded by the residues R219 and Y75, belonging to two different VP1 units. It also showed a direct ionic interaction between negative charged carboxylic group of B14 and positive charged guanidino group of arginine 219. In conclusion, B14 is a novel early-stage inhibitor which targets viral protein VP1. Additional studies are ongoing to determine in further detail the mechanism of action of the compound.

New insights into the Interaction between Pyrrolyl Diketoacids and HIV-1 Integrase Active Site.

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Despite the long lasting fight against AIDS, several therapy failures have enlighted the need of new effective inhibitors.[1] The latest class of drug approved is active site inhibitors of Integrase (IN), a key enzyme in the HIV-1 replication cycle. This class is currently deeply investigated in the attempt to find more potent agents to counteract the emergence of multi-drug resistant HIV-1 strains. Diketo acid (DKA) derivatives are among the first compounds reported to interact with the Mg^{2+} cofactors within the IN active site. Interestingly, some DKAs were also reported to inhibit another emerging anti HIV-1 target, RNase H.[2] Recently, a series of pyrrolyl DKAs was reported to inhibit IN within the nanomolar range and HIV-1 replication.[3] Herein, four promising DKAs were chosen as chemical tools to investigate their interaction with the IN active site and to study their mechanism during HIV-1 replication. Cell culture studies confirmed IN as the DKA target in cell based assays. Molecular modeling coupled with site-directed mutagenesis studies confirmed their binding interactions within IN, showing, moreover, some differences in their interaction pattern when compared with the first generation of INI such as Raltegravir, by demonstrating full effectiveness against HIV-1 Raltegravir resistant strains. These data provide important insights for the rational optimization of new IN inhibitors. Furthermore, comparative modeling studies on the RNase H active site allowed to better delineate the DKA structural features required to modulate the potency towards either IN and RNase H in order to develop effective dual IN/RNase H inhibitors as new chemical agents against HIV-infection.

[1] Tramontano et al. *Antiviral Res.***2005**, 65, 117-124.
[2] Costi et al. *J. Med. Chem.* **2014**, 57, 3223-3234.
[3] Crucitti et al. *J. Med. Chem.* **2015**, 58, 1915-28.

Cidofovir is active against human papillomavirus positive and negative tumor cells by causing DNA damage as one of its working mechanisms.

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Human papillomavirus (HPV) causes cervical cancer and a large fraction of head and neck squamous cell carcinomas (HNSCC). Cidofovir (CDV) proved efficacious in the treatment of several HPV-induced benign and malignant hyper proliferations. To provide a better insight into how CDV selectively eradicates transformed cells, HPV^+ and HPV^- cervical carcinoma and HNSCC cell lines were compared to normal cells for antiproliferative effects, CDV metabolism, drug incorporation into cellular DNA, and DNA damage. Incorporation of CDV into cellular DNA was higher in tumor cells than in normal cells and correlated with CDV antiproliferative effects, which were independent of HPV status. Increase in phospho-ATM levels was detected following CDV exposure and higher levels of γ-H2AX (a quantitative marker of double-strand breaks) were measured in tumor cells compared to normal cells. A correlation between DNA damage and CDV incorporation into DNA was found but not between DNA damage and CDV antiproliferative effects. These data indicate that CDV antiproliferative effects result from incorporation of the drug into DNA causing DNA damage. However, the anti-tumor effects of CDV cannot be exclusively ascribed to DNA damage. Furthermore, CDV can be considered a promising broad spectrum anti-cancer agent, not restricted to HPV^+ lesions.

The interferon-inducible DNA-sensor protein IFI16: a key player in the antiviral response.

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Intrinsic immune defenses are active before a pathogen enters a cell. This first-line of antiviral defense is mediated by a variety of constitutively expressed cell proteins collectively termed "Restriction Factors" (RFs), and they form a vital element of the immune response to virus infections. Among the RFs IFI16, a member of the IFN-inducible PYHIN-200 gene family displays a multifaceted activity due to its ability to bind to various target proteins and, in turn, to modulate a variety of cell functions including proliferation, differentiation, apoptosis/pyroptosis, senescence, and inflammation. We have recently demonstrated that IFI16 restricts HCMV replication by down-regulating viral early and late but not immediate-early mRNAs and their protein expression. However, viruses are known to evolve numerous strategies to cope and counteract such restriction factors and neutralize the first line of host defense mechanisms. Our studies demonstrate that during early stages of infection, IFI16 successfully inhibits HCMV replication by down-regulating UL54 activity. However, in late stages HCMV mislocalizes IFI16 into the cytoplasmic viral assembly complex (AC) and finally entraps the protein into mature virions. Together, these data demonstrate that IFI16 is a restriction factor of HCMV replication and clarify the mechanisms HCMV relies on to overcome IFI16 viral restriction.

Counteract influenza A by blocking PA-PB1 protein-protein interaction with the aid of molecular modelling.

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Influenza is a seasonal disease commonly known as "flu" is an important health burden responsible for important consequences on the global morbidity, mortality and economy.[1] It is caused by viruses of the Orthomyxoviridae family that possess a ribonucleic acid (RNA) genome and infect vertebrates. Among the three genera, influenza A is responsible of severe upper respiratory diseases in humans that occur seasonally with epidemic and sometimes pandemic proportions.[2] The need of annual updating of the anti-influenza vaccine and the rapid emergence of viral strains resistant to available therapy make the need for antiviral drugs that exploit novel mechanisms of action urgent.[3] The viral RNA polymerase (RdRp) is a heterotrimer essential for viral replication and less prone to mutations than current targets. Moreover, the interaction between two of its three subunits (PA, and PB1) is essential for RdRp activity and viral infectivity, making the disruption of this complex a promising drug design strategy.[4]Through a virtual screening procedure we identified a novel class of 3-cyano-4,6-diphenyl-pyridines that inhibit the PA-PB1 interaction.[5] According to our model, these molecules bind to PA in the site of binding of PB1, superposing very well with its N-terminal residues. We chemically modified this scaffold aiming the optimization of the compounds' activity and the understanding of the structure-activity relationships. Non-cytotoxic compounds with both the ability of disrupting the PA-PB1 interaction and antiviral activity were identified and their mechanism of action was clarified with molecular modelling simulations.[6]

Characterization of the antiviral mode of action of anisomycin against dengue virus.

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Anisomycin is an alkaloid that was proved to inhibit the multiplication of Japanese encephalitis virus. Inthis work we investigated the antiviral activity of anisomycin against dengue virus (DENV) in cell cultures. Cell viability was assessed by the MTS method and the 50% cytotoxic concentration wasdetermined (CC50). Antiviral activity was evaluated by a virus yield inhibition assay and the 50%effective concentration (EC50) was determined and the selectivity index (SI) was calculated as theratio CC50/EC50. Anisomycin was active against the four DENV serotypes in Vero cells with SI valuesof 232.3, 171.9, 216.9 and 87.3 for DENV-1, -2, -3 and -4, respectively, and in all cases a 99.99% ofvirus yield inhibition was achieved at 200nM concentration. On the contrary, anisomycin was inactiveagainst poliovirus and Junín virus. A potent inhibition was also observed in a range of multiplicity ofinfections from 0.1 to 50 PFU/cell and in human cell lines. Time of addition experiments demonstratedthat 99,99% inhibition of virus yield was achieved when anisomycin was added at 1, 3, 5 or 8h postinfection(p.i.), while the inhibitory effect decreased when the compound was added at 12 h p.i andnon-significant inhibition was observed when the addition of the compound was done at later times p.i.Kinetics of viral entry was not affected by anisomycin whereas a strong inhibition of viral proteinexpression, assayed by indirect immunofluorescence, and RNA synthesis, determined by RT-real timePCR, was detected. Altogether our results indicate that anisomycin is a selective and potent inhibitorof DENV multiplication that would mainly affect viral macromolecular synthesis.

Application of cell based phenotypic assays for the evaluation of novel HIV-1 inhibitors targeting viral and host proteins

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Current antiretroviral regimens can successfully halt HIV-1 replication in the majority of treated patients, resulting in chronicization of asymptomatic disease and improvement of the quality of life of infected patients. However, the emergence of drug resistance can still compromise treatment efficacy and reduce the therapeutic options, thus the need of new antivirals active against resistant strains is still required. We applied a homemade phenotypic approach to evaluate the antiviral activity of two novel classes of candidate HIV-1 inhibitors targeting the viral nucleocapsid protein p7 (NCp7) and the human ATPase/RNA helicase DDX3 protein[1], respectively. IC50 values were calculated through a phenotypic assay consisting in a first round of infection of MT-2 cells in presence of serial dilutions of the investigational compounds. After 72 hours, MT-2 supernatants were used to infect the reporter cell line TZM-bl and after 48 hours RLUs were counted and elaborated through the GraphPad software to calculate IC50 values. Since NCp7 is involved in several steps of HIV-1 life cycle, we performed a single cycle infection assay using TZM-bl cells to evaluate the inhibition of early steps of HIV replication by NCp7 inhibitors. These assays allowed the identification of several compounds with antiviral activity in the low micromolar range against both wild type virus and strains carrying most common patterns of resistance mutation selected by currently approved HIV-1 drugs. Further experiments aimed at characterizing the mechanism of action of the candidate anti-HIV compounds are in progress.

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Future Perspectives in Antiviral drug delivery.

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The global impact of viral infections with the emergence of new viruses and the development of resistance to current drugs translate into a continuous need for new antiviral therapeutics and approaches. In the overall scenario, Nanotechnology offers new therapeutic ways for attacking viral deseases and for improving treatment success. Nanoparticle-based systems, when combined with classical antiviral agents, can increase the antiviral bioavailability with improved solubility and stability of the therapeutics. Moreover, they permit the delivery of the antiviral drug to the specific target site restricting the adverse side effect, reducing the intake frequency and shorten the time of treatments, potentially rendering the treatment more cost-effective. While the future of nanoparticle-based therapy is promising, there are still many challenges and barriers to achieve its full potential, as for example a deep exploration of the toxicological and the bioelimination aspects of nanocarriers to ensure safe manufacture and use of nanomaterials [1].This report will present an assortment of nanomaterials that can be used as potential candidates for antiviral delivery:

- Superparamagnetic ferrite nanoparticles synthetized by microemulsion technique [2];
- PEGylated magnetite-silica nanocomposites obtained by High Energy Ball Milling (HEBM) [3];
- Silica-based mesoporous materials prepared by liquid-crystal template method and HEBM [4].

It is the starting point for a discussion within biologists, medicinal and inorganic chemists on Innovative Approaches for Antiviral drug delivery.

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Antiviral activity and cytotoxicity of selected essential oils.

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Essential oils (EO) have been reported to possess versatile biological activities, including antibacterial, antifungal, antiprotozoal and antiviral activity. In this study commercially available essential oils from carrot seed (CAR), clove (CLO), eucalyptus (EUC), citronella (CIT) and pine (PIN) were tested for cytotoxic properties and antiviral activity against HHV-1. Furthermore, the influence of EO on cell cycle was evaluated.
Cytotoxicity was assessed on VERO (green monkey kidney) and FaDu (human pharynx squamous cell carcinoma) cell lines using the MTT method. EO in non-toxic concentrations were incubated with HHV-1 infected Vero cell line until the cytopathic effect (CPE) was observed in the positive control. Subsequently, the virus titre was measured using end-point dilution assay. Furthermore, the qPCR was used to determine the amount of viral DNA in samples. The influence of EO on cell cycle was tested using NucleoCounter NC-3000 image cytometer. The composition of EO was tested with the use of GC/MS. Moreover, the stability of selected EO in culture media during incubation was tested.
EUC and CIT showed lowest toxicity on VERO (IC_{50} 277.6 and 196.9 µg/ml, respectively). EUC showed selective toxicity towards FaDu (IC_{50} 49.2 µg/ml, SI=5.64). The highest toxicity on FaDu was observed for pine oil (IC_{50} 6.2 µg/ml, SI=6.69). Among tested EO, only CIT in the concentration of 75 and 125 µg/ml inhibited CPE formation in HHV-1 infected VERO cell line, resulting in the decrease of the virus titre by 0.99 and 5.02 log, respectively. The results of qPCR suggest that CIT inhibit late stages of HHV-1 replication. No influence of CIT on cell cycle was detected.

A small Stem-Loop Structure of the Ebola Virus Trailer interacts with HSPA8 and is essential for Ebola Virus Replication.

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Ebola virus (EBOV) is a single-stranded negative-sense RNA virus belonging to the *Filoviridae* family. The leader and the trailer noncoding regions of the EBOV genome likely regulate EBOV transcription, replication, and progeny genome packaging. We investigated the *cis*-acting RNA signals involved in RNA-RNA and RNA-protein interactions that regulate replication of eGFP-encoding EBOV minigenomic RNA and identified heat shock cognate protein family A (HSC70) member 8 (HSPA8) as an EBOV trailer-interacting host protein. Mutational analysis of the trailer HSPA8 binding motif revealed that this interaction is essential for EBOV minigenome replication. The RNA secondary structure of the EBOV minigenomic RNA was evaluated. Our results indicate the formation of a small stem-loop composed of the HSPA8 motif, a 3' stem-loop (5'18882-18902-3') that is similar to a previously identified structure in antigenomic RNA, and a panhandle domain involving a trailer-to-leader interaction. Results of EBOV minigenome assays and an EBOV reverse genetic system rescue support a role for the panhandle domain and the HSPA8 motif 1 in virus replication. Our studies provide insight into EBOV RNA tertiary structure, identify unique RNA-RNA and RNA-protein interactions, and establish a structural map to aid the design of RNA motif-based antiviral compounds.

The A-type Proanthocyanidins (PACs-A) of a novel cranberry extract inhibit Herpes Simplex Type 1 and Type 2 infections.

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In the absence of efficient preventive vaccines, topical microbicides offer an attractive alternative in the prevention of Herpes simplex type 1 (HSV-1) and type 2 (HSV-2) infections. Because of their recognized anti-adhesive activity against bacterial pathogens, cranberry (Vaccinium macrocarpon Ait.) extracts may represent a natural source of new antiviral microbicides. However, few studies have addressed the applications of cranberry extract as a direct-acting antiviral agent. Here, we report on the ability of the novel cranberry extract Oximacro® and its purified A-type proanthocyanidins (PACs-A), to inhibit HSV-1 and HSV-2 infection in vitro. Analysis of the mode of action revealed that Oximacro® prevents attachment of HSV-1 and HSV-2 to target cells. Further mechanistic studies confirmed that Oximacro® and its PACs-A directly interact with the viral envelope glycoproteins gD and gB on virion surface, thus resulting in the loss of infectivity of HSV particles. Moreover, Oximacro® completely retained its anti-HSV activity even at acidic pHs (3.0 and 4.0) and in the presence of 10% human serum proteins; conditions that mimic the physiological properties of the vagina - a potential therapeutic location for Oximacro®.
Taken together, these findings indicate Oximacro® as an attractive candidate for the development of novel microbicides of natural origin for the prevention of HSV infections.

Characterisation of a mouse model for the study of HDV chronic infection.

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One of the most important limitations for the experimental study of hepatitis delta virus (HDV) is theabsence of small animal models featuring all the characteristics of human infection [1-5]. Here wedescribe a new mouse model of HDV infection that reproduces the main characteristics of the humanviral infection. We used recombinant adeno-associated viruses (AAV) as Trojan horses for both HDVandHBV replication-competent genomes. AAV-HDV infection resulted in HDV replication invitro and in vivo with the formation of HDV genomes and antigenomes and in the expression of shortand long HDV antigens. In the presence of HBV, HDV infectious particles were detected in serum andHDV replication was more efficient. Furthermore, as observed in patients, co-infection was associatedwith the development of a more severe liver pathology[6] that manifested in the upregulation of genesknown to be involved in liver damage. Significant differences in viral replication, antigen expression aswell as liver damage were observed between immune-competent and immune-deficient mice, hintingat the important role played by the adaptive immune response in the viral life cycle and in thepathogenesis of this infection. Interestingly, HDV replication evoked a sustained type I IFN responsewhich was significantly ameliorated in immune-deficient mice and completely absent in MAVS deficientmice. Indicating that MAVS signalosome play a main role on HDV-mediated activation of the innateresponse and that this response is amplified by the immune system.

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Role of receptor tyrosine kinases and associated gangliosides in the influenza virus replication cycle.

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Like all viruses, influenza virus crucially depends on a wide array of host cell factors for its replication. Targeting these cellular factors is a potentially relevant antiviral strategy, since cellular proteins are less likely to change and consequently, resistance against this type of antiviral-compounds is less likely to emerge. In this project, we focus on inhibitors of cellular protein kinases as potential influenza virus inhibitors. Through screening of a protein kinase inhibitor library, we identified Ki8751 as a compound with robust antiviral activity against a broad range of influenza viruses and low cytotoxicity. Ki8751, a known inhibitor of specific receptor tyrosine kinases (RTKs), was shown to act during the viral entry phase but had no effect on virus binding to the cells. In parallel, we observed that influenza virus entry is increased in CHO-K1 cells when compared to CHO-wild type cells. CHO-K1 cells lack complex gangliosides (e.g.GM1) but do express the GM3 ganglioside, which has an either positive or negative role in RTK signaling pathways[1]. Hence, we are verifying whether the antiviral activity of Ki8751 is based on inhibition of a specific type of RTKs that is positively regulated by GM3.Using a panel of Chinese hamster ovary transfectant cell lines, we further found that influenza virus replication is increased in CHO cells that express the vascular endothelial growth factor receptor 2 (VEGFR2). Strikingly, this effect was located at the stage of viral RNA synthesis and not viral entry. We hypothesize that this could be related to a link between intranuclear VEGFR2, the Sp1 transcription factor and/or the cytoprotective protein SOD[2,3,4].

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Poster abstracts

Skin irritation of SPD-incorporated ointment on BALB/c Mice and its Antiviral Activity against Cutaneous Herpes infection.

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Styrylpyrone derivative (SPD) from *Goniothalamus umbrosus* has been found to be a potent antiviral agent against Herpes Simplex virus type-1 (HSV-1). Its proposed mechanism is associated with apoptosis and cell cycle arrest, which is different from current commercial antivirals. This study was aimed to assess possible skin irritation of SPD-incorporated ointment towards BALB/c mice and to evaluate its antiviral activity against cutaneous herpes infection. Several concentration of SPD (1.25-20% w/w) was incorporated into ointment base and applied onto nude dorsal area of BALB/c mice once daily for 7 consecutive days. Ointment base (without SPD) and 5% potassium hydroxide were used as negative and positive control respectively. It was found that the highest concentration of SPD-incorporated ointment without irritation was 1.25%. Severe and moderate irritation was observed in 10% and 20%-SPD ointment. Therefore, 1%-SPD ointment was chosen to be used in antiviral evaluation. Cutaneous herpes infection was initiated by inoculating 106 plaque forming unit of HSV-1 onto abraded nude dorsal area of BALB/c mice. Two types of treatments were administered; treatment given at 6 hours post infection (hpi) and at 24 hpi. Both treatments were administered twice daily for 6 consecutive days. Infected mice treated with Acyclovir cream or with ointment base were used as positive and negative control respectively. Results showed that treatment at 6 hpi able to reduce lesion formation, compared to non-treated group. In conclusion, SPD-incorporated ointment was found to be non-irritant at concentration of 1.25% and below. Antiviral activity of 1%-SPD ointment was observed when treatment was administered at 6 hpi.

In silico identification of new natural ligands as potential HIV-1 Integrase agents.

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The human immunodeficiency virus type1 (HIV-1) integrase (IN) is an essential enzyme in the life of the virus and is an attractive target for the development of new drugs useful in the acquired immunodeficiency syndrome multidrug therapy. HIV-1 IN inserts viral DNA into the host genome through two sequential reactions. The first one, termed "3'-processing (3'-P), involves the cleavage of the 3'-dinucleotides from viral DNA; the second reaction, defined "strand transfer" (ST), involves the subsequent insertion of the processed ends of viral DNA into the host DNA. The IN strand transfer inhibitors (INSTIs) represent the newest class of anti-AIDS drugs approved by the Food and Drug Administration (FDA). However, in infected patients, the emergence of viral resistance is a limitation for all anti-HIV therapeutics, including INSTIs as raltegravir (RAL), elvitegravir (EVG) and dolutegravir (DTG) [1].Starting from 1WKN [2] Protein Data Bank (PDB) structure, molecular dynamics (MD) and docking simulations were carried out. Such theoretical model is a full-length HIV-1 IN dimer complexed with the viral-DNA (Figure 1). A virtual screening of natural databases was performed against the HIV-1 IN, allowing us to identify some promising hits characterized by a better theoretical binding affinity if compared to that of the reference compounds. The results obtained from this study could shed light on the activity of a new series of lead compounds as potential HIV-1 IN inhibitors and could be useful in the rational drug design.

Antiviral effect of Hydroxyurea on Parvovirus B19 replication.

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Human Parvovirus B19 (B19V) is a ssDNA virus responsible of different diseases but still without an antiviral therapy. The acyclic nucleotide analogue *Cidofovir* is the only compound for which an inhibitory activity has been recently established against B19V *in vitro* [1]. Since CDV has a difficult uptake, the present study had the purpose to investigate *Hydroxyurea (HU)* as a new putative anti-B19V compound. HU is usually used in the treatment of various cancers and myeloproliferative disorders; in addition an anti-HIV activity was discovered due to its ability to induce cell cycle arrest through inhibition of ribonucleotide reductase [2]. Our experiments were carried out using two cellular systems: UT7/EpoS1 cell line and *ex vivo*-expanded EPCs (erythroid progenitor cells) [3]. They were infected with B19V at a multiplicity of infection of 10^4 viral genomes/cell and were cultured in presence of HU at different concentrations (range: 0,1 μM – 50 mM). B19V replicative activity was evaluated and compared at 2 hpi and 48 hpi by quantitative PCR assay. Moreover cellular proliferation and viability within HU were monitored in both model systems. Results suggested an *in vitro* dose-dependent inhibitory action of HU towards B19V replication. In fact the antiviral effects (expressed as EC_{50}) valued of 139 μM for UT7/EpoS1 and 175 μM for EPCs, respectively. Besides a specific activity of the compound against B19V could be hypothesized, since HU showed cytostatic effects at higher concentrations with values of 457 μM and 491 μM, respectively, while a cytotoxic effect was only showed in EPCs at HU concentrations > 500μM.
In conclusion, our studies have demonstrated the strong antiviral activity of HU towards B19V, opening the possibility to multiple uses of this drug *in vivo*.

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Purification and on-column refolding of full-length EBOV VP35 with good dsRNA binding activity measured in a simple fluorescence assay.

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Ebola virus (EBOV) VP35 is a structural protein of the viral nucleocapside and a cofactor in viral replication and transcription[1] that also plays a key role in infection during Ebola Virus disease (EVD). In fact, VP35 is able to inhibit the induction of the IFN-α/β expression that follows viral infection through multiple mechanism[2,3], among which the VP35 binding to the viral dsRNA is crucial to block the RIG-I pathway and its virus-induced phosphorylation and activation of interferon regulatory factor 3 (IRF-3)[4]. We previouslypresented, for the first time, the expression and purification in a prokaryotic system of the functionally active, recombinant full length VP35 protein of EBOV[5] and reported the establishment of a biochemical assay to determine VP35-dsRNA binding activity[6]. Those methods presented two major limitations: the low VP35 yield and the use of radioactive labelled dsRNA. We report a new methodology to improve the VP35 yield by urea denaturation and refolding. Furthermore, we developed in 96-well Nickel coated plates a new *in vitro* biochemical assay to quantify VP35-dsRNA binding activity using a 5'-fluorescein-dsRNA as substrate. We measured the equilibrium dissociation constant for VP35-dsRNA binding by this fluorescence based assay obtaining VP35 binding parameters similar to those reported in previous studies[5]. We finally validated the assay to screen EBOV VP35 inhibitors by assessing the inhibitory ability of an in vitro transcribed 500 bp dsRNA.

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Inhibition of the HIV-1 integrase-LEDGF/p75 interaction by 5,6-dihydroxyindole-2-carboxylic acid derivatives.

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The HIV-1 integrase (IN) enzyme catalyzes two distinct reactions termed 3'-processing and strand transfer, which allow for the integration of the viral DNA into the host chromosome. HIV-1 IN functions as a multimer and forms the stable synaptic complex (SSC) with the viral DNA ends. Among the cellular factors involved in the integration process into the host DNA there is the human lens epithelium-derived growth factor LEDGF/p75, a nuclear protein that promotes tethering of the SSC to chromatin by establishing specific interactions between its IN-binding domain and the IN dimer. The need for HIV-1 IN inhibitors targeting allosteric binding pockets is based on the observed emergence of IN strand transfer specific drug-resistant mutations upon clinical use of the approved IN inhibitors. For this reason, we reconsidered and more carefully evaluated the biological activity of previously discovered 5,6-dihydroxyindole-2-carboxylic acid (DHICA) derivatives by Homogeneous Time Resolved Florescence (HTRF) assays. In particular, DFC 18 derivative potently inhibited IN-LEDGF/p75 interaction and IN-IN dimerization with IC_{50} values of 0.18 and 0.8 μM, respectively. In contrast, DFC 13 is less potent than DFC 18 on IN-LEDGF/p75 interaction and IN-IN dimerization, but is able to block the HIV-1 replication. Further studies are ongoing to better understand the mechanism of action of this class of compounds.

Combination of anti HIV-1 siRNAs and a fusion inhibitor for genetic treatment of AIDS.

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Despite the remarkable success of highly active antiretroviral therapy (HAART) in lowering the plasma viral load in patients with HIV-1 infection and in slowing down the disease progression, many obstacles still remain, including the generation of drug-resistant viral species, the difficulty in eradicating latent reservoirs and the high costs of the therapy. The development of complementary strategies to completely eradicate or to control HIV-1 infection without daily drug intake is therefore a priority. In this context, anti HIV-1 gene therapy based on intracellular immunization of autologous T cells or their progenitors, i.e. the $CD34^+$ hematopoietic stem cells, resistant to infection, appears a particularly promising approach to repopulate the immune system.In order to interfere with different steps of viral replication, we previously developed a series of self-inactivating lentiviral vectors expressing multiple small interfering (si)-RNAs targeting the CCR5 cellular gene as well as vif and tat/rev viral transcripts, under the control of different RNA polymerase III promoters (U6, 7SK, H1). The use of a single RNA polymerase III promoter driving the expression of a sequence giving rise to three siRNAs directed against the selected targets (e-shRNA) was also investigated. Two effective anti-HIV combinatorial vectors that conferred protection against R5 and X4 tropic virusesin human primary CD4+ T lymphocytes were identified. Further modification of the combinatorial vectors were accomplished by the inclusion of a membrane-anchored peptide (maC46), which has been shown to efficiently block the entry of both CXCR-4 and CCR5-using viruses. The maC46 was inserted under the transcriptional control of the human Elongation Factor 1 promoter. Additionally, an optimized version of the Woodchuck hepatitis virus post-transcriptional regulatory element (WPRE*) was also inserted in the new generation of lentiviral vectors. The antiviral activity of the optimized vectors was assessed in different cellular models. Overall our results contribute to gain further insights in the design of combinatorial gene therapy approaches against HIV-1 for clinical application.

Development and validation of a luciferase reporter gene assay to quantify Ebola Virus VP24 inhibition of IFN signaling.

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The interferon (IFN) system is the first line of defense against viral infections. IFN signaling triggers nuclear transport of P-STAT1, which occurs via karyopherin-α (KPNA) and lead to the expression of IFN stimulated genes (ISGs) with antiviral activity. Ebolavirus VP24 protein acts binding KPNA to inhibit P-STAT1 nuclear transport (1,2,3) and it can also directly bind STAT1(4). The suppression of IFN signaling by VP24 is a critical event in the pathogenesis of the infection. Being a key factor for EBOV virulence (5), VP24 is a potential target for drug development. Since no licensed drugs target VP24, the identification of molecules able to inhibit VP24, restoring and possibly enhancing the IFN response, is a goal of concern. Accordingly, we developed a cell-based drug screening assay able to quantify IFN induction, its inhibition by VP24 and the antiviral effect of compounds. HEK293T cells are transiently transfected with a luciferase reporter gene construct (pISRE-luc) driven by the promoter element of ISGs genes. Stimulation of cells with human recombinant IFN-α activates the IFN signaling cascade leading to the expression of ISRE, quantified by a luminescent signal. To adapt this system to test a large numbers of compounds, we performed it in 96-well plates. We validate the assay analyzing different conditions: timing for optimal relative luciferase unit, timing of transfection and stimulation, concentrations of plasmids, IFN and compounds were optimised by experimentation to detect results with accuracy and precision as proved through statistical verification, and to ensure repeatability and reproducibility in the assay.

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Traditional Chinese Medicine as a source of molecules for counteracting Ebola virus VP35 protein inhibition of the interferon cascade.

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Ebola virus (EBOV) is causative agent of severe viral hemorrhagic fever in humans and non-human primates, with a case fatality rate of up to 88% in human outbreaks. Last Ebola virus disease (EVD) outbreaks confirmed that EBOV epidemics are highly unpredictable and the need to identify small molecules that can counteract EVD is a global health priority. Suppression of innate immune responses during EBOV infection contributes to disease severity. EBOV encodes VP35 protein, potent suppressor of RIG-I-like receptor (RLR) signaling and interferon-α/β (IFN-α/β) production by several mechanisms (1, 2, 3, 4), including direct binding to double stranded RNA (dsRNA) (2, 4). Hiding RLRs recognition sites on dsRNA by binding to phosphate backbone and capping dsRNA ends, EBOV VP35 suppresses host IFN-α/β production (2, 3, 5). A miniaturized luciferase reporter gene assay was applied in order to screen molecules that: i) can activate and potentiate IFN cascade and the subsequent immune response; ii) can interfere with EBOV VP35 viral inhibition of the immune response (7). Traditional Chinese herbal medicine (TCHM), the most important component of the traditional Chinese medicine system, has long been used for its multiple combinations of compounds in the form of processed natural products (8, 9). In reason of that, a number of selected herbal extracts derived from TCM were assessed. We identified a *Scutellaria Baicalensis* extract significantly capable in restoring the RIG-I-mediated IFN-b production inhibition induced by EBOV VP35. This result opens the field to the possibility of being able to identify an ideal intracellular partner EBOV VP35 protein and implement new and effective countermeasures against EVD.

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Optimizing potency and metabolic stability of [1,2,3]triazolo[4,5-*d*]pyrimidin-7(6*H*)-ones as chikungunya virus replication inhibitors.

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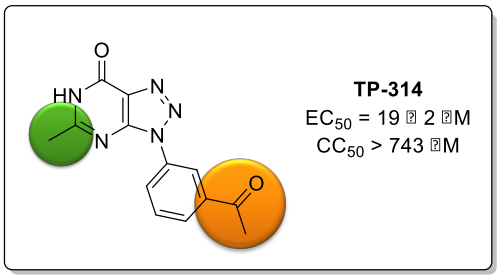
Chikungunya fever (CHIKF) is an arboviral disease caused by the Chikungunya virus (CHIKV). Although traditionally transmitted to humans by *Aedes aegypti*,located in certain regions of Africa and Asia, the adaptation of the virus to *Aedes albopictus*, a mosquito species with an almost worldwide distribution, has contributed to the geographical spread of this virus in the past decade.[1] CHIKF is characterized by fever, rashes, myalgia and painful arthralgia that lasts for months or even years. Nowadays no effective treatment (vaccine or drug) against CHIKV is available[2]. Our research group, together with the virology lab at Rega Institute, has been fully involved in the identification and study of small molecules able to inhibit CHIKV replication. We have identified a sample coded **TP-314** as an inhibitor of CHIKV replication (EC_{50} = 19 μM) at non-toxic concentrations (CC_{50}> 743 μM).[3] We have now undertaken the synthesis and anti-CHIKV evaluation of novel series of compounds with two main objectives: to improve the antiviral potency of this family of compounds and to increase the metabolic stability of our initial hit compound.



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Analogues of inorganic pyrophosphate as multitarget HIV replication inhibitors.

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Despite the undoubted success in the development of human immunodeficiency virus type 1 (HIV-1) therapyby means of a bundle of reverse transcriptase (RT), integrase (IN), protease, viral entry and fusion inhibitors, this infection still remains a significant health threat. One of the current problems associated with HIV therapy is phosphorolytic excision of 3'-terminal nucleotide analogs (e.g. AZT, 3TC) and the emergence of drug-resistant virus variants containing specific RT mutations called thymidine analogue mutations (TAMs, M41L, D67N, K70R, T215Y, K219Q), which enhance pyrophosphorolytic excision of NRTIs.
Herein, we synthesized methylenebisphosphonates 40 BPs, several of which simultaneously inhibited phosphorolytic activity of native and drug-resistant forms of HIV-1 RT, RT-catalyzed elongation and two enzymatic activities of the integrase. In addition BPs exhibited low anti-RNase H activity. For effective inhibition, BPs should be comprised of three elements: the Mg^{2+}-coordinating methylene bisphosphonate backbone, the aromatic halogenated pharmacophore linked to the backbone through the inert aliphatic linker. The most active was a bis-dichlorophenyl containing bisphosphonate 47, which suppressed RT and IN at low micromolar concentrations. Some compounds didn'tsuppress double mutant RT bearing K103N and Y181C. These mutations are associated with resistance to non-nucleoside RT inhibitors (NNTRIs). BP 47 was active against the three RT variants: the wild type, double mutant and RT bearing thymidine analogue mutations. 47 appeared to be a non-competitive inhibitor of RT-catalyzed elongation.

Identification of Antagonists to Human Immunodeficiency Virus (HIV) Entry Using HIV-Env Pseudotyped Virus.

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At present, the AIDS-inducing HIV remains a significant medical burden worldwide, with ~ 37 million carriers and 2-3 million new infections occurring annually. Despite the use of HAART treatment, drug resistance and the lack of a cure and preventive vaccine will likely project further HIV-associated morbidity/morality associated in the near future. These issues highlight the importance of developing additional antivirals, such as entry inhibitors, to support the management of HIV infections. Viral pseudoparticle systems can be used to mimic the early viral entry steps and adapted for the discovery of viral entry inhibitors. In this study, a reporter-based pseudoparticle system expressing HIV-Env (HIVpp) was used in conjunction with specific viral entry assays to identify candidate anti-HIV agents from a natural product library. Results revealed that the methanolic extracts from *Ganoderma lingzh* and *Taiwanofungus camphorata*, the plant alkaloid berberine, and the nanoformulated terpenoid NP-1 all inhibited HIVpp infection of Jurkat cells by >60% at non-cytotoxic doses, with NP-1 exhibiting the strongest antiviral activity (>80% inhibition). Subsequent analysis showed that NP-1 could antagonize the HIVpp infection in a dose-dependent manner, and its selectivity index was determined to be 19.3 (CC_{50} = 257.4 μM / EC_{50} = 13.3 μM). Further mechanistic studies of NP-1's effect against distinct steps of the HIVpp entry (pre-attachment free virion, virus binding to host cell surface, and post-attachment entry/fusion phase) are underway. The HIVpp system should be useful to help expand the scope of candidate HIV antivirals for prophylactic and/or therapeutic application.

Chemical and biological analysis of traditional Chinese medicines.

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The constituents in plants, such as phenolics, tannins and flavonoids exhibited the abilities to scavenge free radicals1. Free radicals accelerated viral mutation by increasing oxidative damage. Free radical scavengers could regulate immune systems and reduce the virus infections induced pathogenicity2,3. Traditional Chinese medicines (TCM) revealed antibacterial infections and treatment of human immunodeficiency virus and hepatitis B virus. In the present study, we evaluated the phenolic and flavonoid contents as well as the free radical scavenging activities of TCM.Ten species of TCM were extracted with boiling water. Total phenolic and flavonoid contents of the extracts were determined by the Folin-Ciocalteu and aluminum chloride colorimetric methods, separately. Free radical scavenging activities were analyzed using DPPH, ABTS, and superoxide radical scavenging assays.In chemical analysis, IT-2, IT-4 and IT-10 extracts showed higher phenolic and flavonoid contents (more than 100 mg gallic acid and rutin equivalent/g of extract). In free radical scavenging activities, IT-1 and IT-8 exhibited superoxide (93.56%) and ABTS (90.91%) radicals scavenging activities, respectively. IT-2 exhibited DPPH and ABTS radicals scavenging activities (98.15% and 94.49%). IT-4 and IT-10 exhibited DPPH (93.58% and 88.84%), ABTS (97.81% and 95.04%) and superoxide (86.94% and 72.33%) scavenging activities. IT-2 (worm-expelling medicine), IT-4 (wind-extinguishing and convulsion-relieving medicine), and IT-10 (exterior-releasing medicine) exhibited good free radical scavenging activities, and contained higher phenolics and flavonoids. They probably have potential to treat virus-induced diseases, and further research is needed.

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Free radicalscavenging activities and chemical contents on prescriptions of traditional Chinese medicines.

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Free radicals and oxidative stress have been implicated inpromoting the pathogenesis of infectious diseases caused by viruses[1]. Phenolics and flavonoids were types of phytochemicals displayed viral inhibition, such as influenza viruses, hepatitis B virus, and HIV[2]. Previous studies reported that traditional Chinese medicineprescriptions, Gan-Lu-Siao-Du-Yin and Long-Dan-Xie-Gan-Tan, exhibited antiviral activities[3,4]. In present study, we evaluated free radical scavenging activities and chemical contents on traditional Chinese medicineprescriptions to estimate their antiviral activities.Ten prescriptions of traditional Chinese medicine (ITP-1 to ITP-10) were decocted with hot water. They were fallen into several categories: heat-clearing, phlegm-resolving, blood-regulating, calming, qi-regulating, and exterior-relieving formulas. Chemical contents of decoctionåsults showed that the total phenolic and flavonoid contents of ITP-3, ITP-6 and ITP-8 were more than 50 mg gallic acid and rutin equivalent per g of decoctions. In free radical scavenging activities, ITP-6 exhibited ABTS radical scavenging activity (91.3%). ITP-3 exhibited DPPH and ABTS radical scavenging activities (67.2 and 97.6%). ITP-8 exhibited DPPH, ABTS and superoxide radical scavenging activities (76.2, 97.5, and 59.2%). To conclude,ITP-3 (blood-regulating formula), ITP-6 (qi-regulating formula) and ITP-8 (exterior-relieving formula) showed higher phenolic and flavonoid contents, as well as free radical scavenging activities. They could be excellent sources of decreasing oxidative stress-induced viral infection in the future.

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SAR studies on a Trp Dendrimer that inhibit Early Steps of theReplicative Cycle of Human Immunodeficiency Virus (HIV) andEnterovirus A71 (EV71).

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Recently, we have reported that dendrimers with Trp on the surface and different central cores, exemplified by compound 1 , with a central pentaerythritol scaffold and 12 Trps on the periphery, have antiviral activity against HIV and EV71 enterovirus, two completely unrelated viruses.1,2 Time-of addition experiments demonstrated that these compounds inhibit early step(s) of the replicative cycle of HIV an EV71, an more specifically they inhibit the entry of these viruses into their target cells. Preliminary activity-relationship studies revealed some structural aspects that were important for the synthesis and antiviral activity of these compounds: (a) a certain degree of multivalency on the periphery is necessary (at least 9 amino acid residues linked to the central scaffold through their amino groups); (b) the possibility of using Behera's amine as spacer and carboxylic acids with different rigidity as central scaffolds. We have now performed modifications on the amino acid moieties of prototype 1 in order to determine the structural features essential for the antiviral activity. In particular novel dendrimers were synthesized in which the Trp of the prototype was replaced by: (a) aromatic (phenyl alanine and tyrosine) and non-aromatic (alanine) amino acids, (b) tryptamine, a "decarboxylated" Trp analogue and (c) N-Me Trp, to assess the importance of a hypothetical hydrogen bond between the NH of the indol moiety and the viral surface. The synthesis and the antiviral activity against HIV and EV71 of these compounds have been reported.

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5'-Phosphonate Prodrugs of AZT: Achievements in the Treatment and Prevention of HIV Infection.

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Despite the numerous drawbacks, 3'-azido-3'-deoxythymidine (AZT, Zidovudin, Retrovir) remains one of the key drugs used in the treatment and prevention of HIV infection in both monotherapy and HAART. A strategy in searching for new effective and safe AZT agents among latent (depot) forms of AZT has yielded its positive results. In particular, AZT 5'-H-phosphonate (Nikavir®, phosphazide) has demonstrated clinical advantages over parent AZT: first and foremost, lower toxicity and better tolerability. It can be effectively used for the prevention of vertical transmission from mothers to babies and as an alternative drug for HIV-infected patients with low intolerance to Zidovudin [1]. Preclinical studies of another phosphonate, AZT 5'-aminocarbonylphosphonate, have demonstrated that it releases AZT when taken orally. Pharmacokinetic studies have shown a prolonged action potential [2]. Preliminary results of clinical trials are promising: AZT 5'-aminocarbonylphosphonate is nontoxic for patients at single and multiple oral doses up to 2000 mg (stage I) and effective in monotherapy at multiple oral doses 1200 mg taken once a day (stage II) [3].

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Structural Evolution of (Z)-4-(2-(2-(2-Oxoindolin-3-Ylidene)Hydrazinyl)Thiazol-4-Yl)Aryl Derivatives: Study and Activity Evaluation towards HIV-1 RT RDDP and RNase H Associated Functions.

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We have recently investigate on the activity of (Z)-4-(2-(2-(2-oxoindolin-3-ylidene)hydrazinyl)thiazol-4-yl)arylderivatives towards the DNA polymerase and ribonuclease H (RNase H) associate activities of human immunodeficiency virus type 1 (HIV-1) [1-4]. This new class of compounds exhibited, not only dual activity towards both HIV 1 RT associated functions, but is also capable to inhibit the replication of the viral genome. On this basis and with the aim to further investigate on the structure activity relationships and on the role of the isatine scaffold on the biological properties of such compounds, we have synthesized a new series of derivatives characterized by the introduction of a methyl substituent of the thiazole ring. This structural modification, although apparently of minor impact on the inhibitor structure, may lead to dramatic changes in the reciprocal spatial orientation of the isatine and of the substituted phenyl in the position four of the thiazole spacer. Moreover the formation of an intramolecular hydrogen bond is suppressed and, therefore, the formation of rotamers along the N-N bond is highly favoured. This aspect may lead to a decrease of activity due to a potential higher energetic cost in the formation of the enzyme-ligand complex, but, on the over hand, could be an advantage when adaptive flexibility is required to adapt the ligand to enzyme mutations. The structural aspect and the biological activity of these new derivatives will be discussed in detail in the poster presentation.

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Structural analysis against Hepatitis C virus polymerase in presence of natural resistance-associated substitutions in isolates from DAA-naïve patients.

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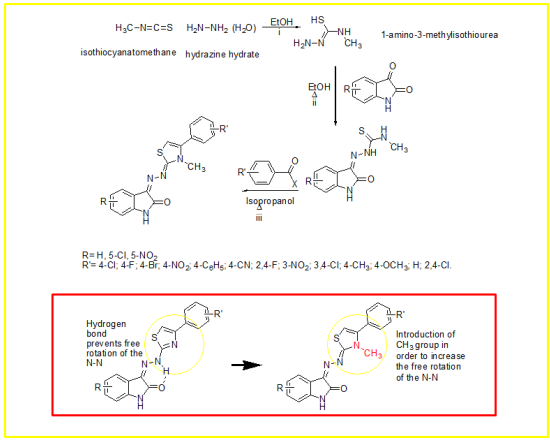
We previously found, in NS5B polymerase from Hepatitis C Virus 1b isolates of six direct-acting antiviral drugs naïve patients, the 316N resistance-associated substitutions (RAS), conferring resistance to Dasabuvir (DSV), in combination with polymorphisms not drug-resistance related [1-3]. Here we investigated the impact of these natural amino acidic variants on apo-polymerase stability and DSV-binding affinity by molecular modelling. *In silico* study started from NS5B deposited in Protein Data Bank [4]. For each isolate, mutants were generated by single-residue replacement and submitted to molecular dynamics simulations (MDs) by Desmond package [5], calculating Root Mean Square Deviation (RMSD). Molecular recognitions were performed by Glide docking [6]. DSV binding free energy (ΔG) *versus* NS5B was predicted by Generalized-Born/Surface Area continuum solvent model of Prime module [7]. After MDs, in the presence of 316N ($RMSD_{316N}$= 3,07 Å) mutation alone, we observed a decreased stability of the NS5B polymerase with respect to the WT ($RMSD_{WT}$= 2,74 Å) indicating a fluctuating conformational state of enzyme. As 316N RAS was associated with the analysed mutations at positions 254, 300, 309, 335 and 338, stability of the enzyme was restored. Docking simulations with 316N RAS (ΔG_{316N}=-50,96kcal/mol) showed DSV thermodynamic profile unfavourable in comparison to WT (ΔG_{WT}=-56,21kcal/mol), while similar profile to WT in presence of polymorphisms was observed. These studies suggest a compensatory role of the natural polymorphisms outside the NS5B active site in presence of the known RAS. Our analysis highlights DSV resistance mechanism towards the 316N-mutated NS5B polymerase.



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5 Desmond Molecular Dynamics System, version 3.4, D. E. Shaw Research, New York, NY, **2013**.
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In silico analysis of resistance mutations naturally occurring in Hepatitis C Virus NS5B polymerase.

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The Hepatitis C Virus (HCV) non-structural protein 5B (NS5B) is an RNA-dependent RNA polymerase (RdRp) required for the replication of the viral genome[1]. Computational analysis was focused on the impact of HCV NS5B naturally occurring mutational patterns on the enzyme stability in the direct-acting antivirals (DAAs)-naïve HCV-infected patients, in order to evaluate the behaviour of the apo-polymerase and the interactions between the novel enzyme sequences and the Non-Nucleoside Inhibitors (NNIs). We used the crystal structure of NS5B apo-polymerase deposited in the Protein Data Bank[2] with the PDB code 1NB4[3]. The mutated models were generated by single-residue replacement and submitted to molecular dynamics simulations (MDs) by means of Desmond[4] package. Ten representative structures from each trajectory were selected as starting receptors for the further docking simulations, performed by using Glide[5] SP protocol. NineteenDAAs-naïve HCV 1b-infected patients were included in the clinical study. HCV genotyping was performed by phylogenetic analysis using maximum likelihood and resistance mutation positions for NS5B sequences were analyzed by Geno2pheno. The C316N mutant, associated to drug-resistance to NNIs[6], was found in six patients. MDs showed that the presence of the C316 played a key role due to the disulfide bridge formation with C366. Our structural results suggested that the C316N decreased the stability of the apo-enzyme and could negatively modulate the binding affinity of the NNIs *versus* the enzyme. The elucidation of this mechanism may be critical to set up an optimal therapeutic approach aimed at improving the drug-resistance profiles.

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Photodynamic Inactivation of *Aedes flavescens* Iridovirus (AfIV) by Fullerene C_{60}.

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There are lots of different photosensitizers, which have been widely investigated as potential antiviral agents. Fullerenes and their derivatives are able to penetrate through cell membranes and they have strong antioxidant, antiviral and antimicrobial properties. Thus, the purpose of this work was to study the effect of photoactivated C_{60} on infectious titer of mosquito iridovirus *Aedesflavescens* (AfIV) in multi host wax-moth larvae *Galleria mellonella*.The most interesting and important finding of the present study is possibility for the photodynamic inactivation of AfIVby using an aqueous solution of C_{60}. Our results showed that after photodynamic inactivation for 30 min C_{60} in a concentration of 0.1 mg/ml reduced the infectious titer of AfIVby 4.0 lg ID_{50}/ml units. In a concentration of 0.01 mg/ml C_{60} reduced theinfectious titer of the mosquito iridovirus by 4.5 lg ID_{50}/ml units. Interestingly, the indexes of infectious titer of AfIV after the photodynamic inactivation for 1 h were not significantly different from those obtained from photodynamic inactivation for 30 min. The useof C_{60} in a concentration of 0.001 mg/ml actually did not influence the infectioustiter of AfIV. The infectious titer of AfIVwas 10^7 lg ID_{50}/ml, that is only 0.5 lg ID_{50}/ml less than in the control group.The presence of C_{60} in a viral suspension, but without the photodynamicinactivation, as well as irradiation of viral suspension in the absence of C_{60}did not influence the infectious titer of AfIV.Solution containing C_{60} plus AfIV was illuminated with visible light for up to 1 h, resulting in a loss of infectivity in wax-moth larvae *Galleria mellonella* of more than 4.5 lgID_{50}/ml. For these reasons, C_{60} may prove useful in the inactivation of viruses in biological systems.

Multi target mode of HIV-1 inhibition by the bioactive molecule Lupeol extracted from the Indian plant Hemidesmus indicus R.Br.

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In the effort of identifying novel therapeutic approaches for the discovery of new anti-infective drugs, the traditional Indian medicine and the use of natural compounds active against various diseases, including viral infections, receive nowadays considerable attention. Hemidesmus indicus R.Br. is known for its ethno-botanical use and its efficacy in the treatment of bone-loss diseases, and, in the Indian traditional medicine, is widely used for the treatment of blood diseases, dyspepsia, loss of taste, dyspnea, cough, poison, menorrhagia, fever and diarrhea. In the last thirty years, many natural substances have shown antiviral activity, but very few of them showed multi target mode of action. For this reason, we assessed the Hemidesmus indicus R.Br. decoction against i) the Human Immunodeficiency Virus type 1 (HIV-1) Reverse Transcriptase (RT)-associated functions and ii) the α-glucosidase activity, with the aim to identify a molecule capable to inhibit with a multiple mode of action different promising targets. Hemidesmus indicus R.Br. decoction has been shown to be active on the inhibition of both RT-associated functions and α-glucosidase activity. Active decoction was fractionated up to obtain the bioactive components which have been isolated, tested and identified as Lupeol, Lupeol Acetate, Caffeic Acid, Chlorogenic Acid and β-amirine acetate. Among these, Lupeol inhibited the HIV-1 RT-associated RNase H function, and the α-glucosidase activity. Our results suggest that this bioactive molecule is a potential lead for further development of drugs with multi target mechanism of action.

Antiviral activity and cytotoxicity of selected essential oils.

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Essential oils (EO) have been reported to possess versatile biological activities, including antibacterial, antifungal, antiprotozoal and antiviral activity. In this study commercially available essential oils from carrot seed (CAR), clove (CLO), eucalyptus (EUC), citronella (CIT) and pine (PIN) were tested for cytotoxic properties and antiviral activity against HHV-1. Furthermore, the influence of EO on cell cycle was evaluated.
Cytotoxicity was assessed on VERO (green monkey kidney) and FaDu (human pharynx squamous cell carcinoma) cell lines using the MTT method. EO in non-toxic concentrations were incubated with HHV-1 infected Vero cell line until the cytopathic effect (CPE) was observed in the positive control. Subsequently, the virus titre was measured using end-point dilution assay. Furthermore, the qPCR was used to determine the amount of viral DNA in samples. The influence of EO on cell cycle was tested using NucleoCounter NC-3000 image cytometer. The composition of EO was tested with the use of GC/MS. Moreover, the stability of selected EO in culture media during incubation was tested.
EUC and CIT showed lowest toxicity on VERO (IC_{50} 277.6 and 196.9 μg/ml, respectively). EUC showed selective toxicity towards FaDu (IC_{50} 49.2 μg/ml, SI=5.64). The highest toxicity on FaDu was observed for pine oil (IC_{50} 6.2 μg/ml, SI=6.69). Among tested EO, only CIT in the concentration of 75 and 125 μg/ml inhibited CPE formation in HHV-1 infected VERO cell line, resulting in the decrease of the virus titre by 0.99 and 5.02 log, respectively. The results of qPCR suggest that CIT inhibit late stages of HHV-1 replication. No influence of CIT on cell cycle was detected.

Effects of D(−) lentiginosine on in vitro HTLV-1 infection.

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We have recently demonstrated that D(-) lentiginosine [-LENT], a non-natural iminosugar with glycosidase inhibitor properties, can cause a preferential, mitochondrial-dependent caspase 3 activation in tumour cells in comparison with non-tumour cells. Moreover, preliminary results suggest that the latter activity could be related to a down-regulation of GLUT-1 receptor expression in tumour cells. Considering the possible role of GLUT-1 in HTLV-1 entry into target cells, we then asked whether [-LENT] could act as a potential drug candidate towards infection caused by HTLV-1. The effects of [-LENT] on PBMC from healthy donors infected in vitro with HTLV-1 by co-culture with irradiated, chronically infected MT-2 or C91/PL cells, were evaluated through real time PCR and flow cytometry analysis. Expression of GLUT-1 was evaluated by confocal microscopy. The [-LENT] inhibited HTLV-1 expression in a dose-dependent fashion, at concentrations in HTLV-1 infected cells. Proliferation of MT-2, C91/PL and HTLV-1-immortalized IL-2-dependent CD4+ cells (CD4/HTLV-1) were inhibited, in comparison with chemotherapeutic agent, after 24 hours of treatment, with an IC50 of 327, 113 and 131 μM, respectively, while stimulated PBMC were inhibited with an IC50 of 170 μM. In addition cell growth of HTLV-1 infected cell lines was more efficiently inhibited after long-term treatment with [-LENT] at the concentration of 5 μM in comparison with stimulated PBMC and 5 μM AZT treated HTLV-1 infected cells. Confocal microscopy studies and flow cytometry analysis showed that [-LENT] inhibited by 50% GLUT-1 receptor expression. In addition in [-LENT] treated cells GLUT-1 was differently distributed in the cytosol in comparison with untreated cells. These data suggest that [-LENT] could protect from both HTLV-1 infection and HTLV-1 immortalization processes by interfering with GLUT-1 and/or GLUT-1 related glucose metabolism of HTLV-1. The use of metabolic inhibitors, in combination or not with other agents, seems an interesting, potential novel strategy against HTLV-1 infection and HTLV-1 associated diseases, owed to presumably low chances for outcome of resistance. Further studies are necessary to verify this hypothesis.

1Minutolo et al. *Cell Death Dis.***2012** Jul 26;3:e358. 2Manel et al. *Oncogene*. **2005**24, 6016–6025.

NOTES

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