International symposium

Non conventional unexpected benefits of protease inhibitors in HAART

Istituto Superiore di Sanità
Rome, October 31, 2003

ABSTRACT BOOK
Edited by
Giuseppina Mandarino and Anna Maria Marella
Dipartimento Malattie Infettive, Parassitarie ed Immunomediate
A relevant issue in the extensive use of Proteases Inhibitors (PI) in the currently adopted therapy for HIV infections is discussed. In the present Symposium different clinical, pharmacological, immunological and microbiological aspects of unconventional, non-antiviral HAART benefits are covered by international and national experts, who focus on PI’s direct effects on previously unsuspected infectious agents, fungal infections and Kaposi sarcoma.

**Key words:** Highly active antiretroviral therapy, Protease inhibitors

Presently, the Istituto Superiore di Sanità, as stated in the Ministerial Decree of 24/01/03, is undergoing a departmental reorganisation. This symposium has been organised by the newly appointed Dipartimento Malattie Infettive, Parassitarie ed Immunomediate (Department of Infectious, Parasitic, and Immune-mediated Diseases), directed by Prof. Antonio Cassone.

This Symposium was also supported by a grant from the Università Cattolica del Sacro Cuore, Rome.
# TABLE OF CONTENTS

Programme ...................................................................................................................... iii

**Session I**  
HAART and immunity ................................................................................................... 3

**Session II**  
HAART direct effects on agents of opportunistic infections ..................................... 9

**Session III**  
Other unconventional HAART benefits ........................................................................ 19

Authors’ index .................................................................................................................. 27
## PROGRAMME

October 31, 2003

<table>
<thead>
<tr>
<th>Time</th>
<th>Session</th>
<th>Title</th>
<th>Presenter(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.00</td>
<td>Introductory remarks</td>
<td>Introductory remarks</td>
<td>E. Garaci</td>
</tr>
<tr>
<td>9.10</td>
<td>Invited lecture</td>
<td>Impact of HAART on morbidity and long-term safety issues: light and shadows</td>
<td>I. Weller</td>
</tr>
</tbody>
</table>

### Session I
HAART AND IMMUNITY
Chairpersons: F. Aiuti, F. Dianzani

<table>
<thead>
<tr>
<th>Time</th>
<th>Title</th>
<th>Presenter(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.40</td>
<td>Immune-reconstitution in HAART</td>
<td>B. Autran</td>
</tr>
<tr>
<td>10.00</td>
<td>HIV-1 protease inhibitors enhance bone marrow progenitor cell activity in normal subjects as well as in HIV-1 infected patients</td>
<td>F. Aiuti, A. Isgrò</td>
</tr>
<tr>
<td>10.20</td>
<td>New antiretroviral drugs</td>
<td>S. Vella</td>
</tr>
<tr>
<td>10.40</td>
<td>Break</td>
<td></td>
</tr>
</tbody>
</table>

### Session II
HAART DIRECT EFFECTS ON AGENTS OF OPPORTUNISTIC INFECTIONS
Chairpersons: A. Cassone, L. Ortona

<table>
<thead>
<tr>
<th>Time</th>
<th>Title</th>
<th>Presenter(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>11.20</td>
<td>Small peptidomimetics inhibiting both HIV-1 and C. albicans aspartic proteases</td>
<td>D. Romeo</td>
</tr>
<tr>
<td>11.40</td>
<td>HAART and candidiasis</td>
<td>A. Cassone</td>
</tr>
<tr>
<td>12.00</td>
<td>HIV protease inhibitors and P. carinii</td>
<td>C. Atzori</td>
</tr>
</tbody>
</table>
12.20 HIV protease inhibitors reduce Cryptosporidium parvum infection in both in vitro and in vivo models
E. Pozio

12.40 The effect of HIV protease inhibitors on C. neoformans virulence and host defenses
A. Vecchiarelli

13.00 HAART and Cryptococcus: effect of indinavir on Cryptococcus neoformans and its protease(s)
C. Mussini

13.20 Lunch

Session III
OTHER UNCONVENTIONAL HAART BENEFITS
Chairperson: G. Majori, M. Moroni

15.00 HAART and proteosome
P.A. Tovo

15.20 HIV-protease inhibitors modulate apoptotic proneness of activated T lymphocytes via a target effect on their mitochondria
W. Malorni

15.40 Anti-angiogenic and anti-tumor activity of HIV protease inhibitors
B. Ensoli

16.00 Modulator activity of PIs in doxorubicin-selected multidrug resistant Kaposi’s sarcoma cell line
M.B. Lucia

16.20 HAART and poverty related diseases: the special case of Malaria
A. Savarino

16.40 Conclusions
R. Cauda
Invited lecture

IMPACT OF HAART ON MORBIDITY AND LONG-TERM SAFETY ISSUES: LIGHT AND SHADOWS

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Department of Sexually Transmitted Diseases, Royal Free & University College Medical School, University College, London

The introduction of Highly Active AntiRetroviral Therapy (HAART) with combinations of at least three drugs in 1996 has led to a dramatic reduction in mortality and morbidity in resource rich countries. Although, a minority of patients still present and are diagnosed with late stage disease many of the problems now facing us are related to the adverse events of therapy. These adverse events include hypersensitivity reactions, hepatitis, gastrointestinal symptoms, lipodystrophy metabolic abnormalities, some of which may be associated with an increased risk of cardiovascular disease and other problems related to known or assumed mitochondrial toxicity. Furthermore, various interactions between the hepatitis viruses and HIV, some related to therapy, have been recognised. The European Medicines Evaluation Agency has taken important initiatives to encourage the post marketing study of some of these adverse events. In addition, there is a heightened awareness of the importance of drug safety in industry and academia with novel approaches in both pharmacovigilance and pharmacoepidemiology.
Session I

HAART and immunity

Chairpersons

Fernando Aiuti, Ferdinando Dianzani
IMMUNE-RECONSTITUTION IN HAART

Brigitte Autran
_Hopital Pitié, Salpêtrière, Parigi_

Not submitted.
HIV-1 PROTEASE INHIBITORS ENHANCE BONE MARROW PROGENITOR CELL ACTIVITY IN NORMAL SUBJECTS AS WELL AS IN HIV-1 INFECTED PATIENTS

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HIV-1 Protease inhibitors (PIs) may improve haematopoietic functions owing to their direct effects on the bone marrow (BM) progenitor cells. In this study we have investigated this hypothesis by evaluating the effect of adding Ritonavir and Indinavir on haematopoietic colony formation assays, cytokine production and stromal cells, in subjects with HIV-1 infection and in seronegative controls. BM samples have been obtained from breastbones of 5 HIV-1 infected patients and from 8 seronegative subjects (controls). The effect of PIs on BM colony formation has been evaluated in short-term cultures by the colony forming cell assay (CFC), with and without the addition of Ritonavir or Indinavir. The growth of the most immature progenitor cells has been evaluated using Long Term Culture-Initiating Cell (LTC-IC) assay with the weekly addition of Ritonavir or Indinavir (10 μM). Stromal cells were also obtained from bone marrow mononuclear cells (BMMCs) cultured in presence or absence of Ritonavir or Indinavir and analysed by immunohistochemistry. At baseline CFC assays in HIV-1 infected patients showed levels of BFU-E, CFU-E, CFU-GM and CFU-GEMM lower than those observed when Ritonavir or Indinavir have been added to the cultures. This finding has been also observed in control subjects, with an increased growth of in vitro colonies after addition of Ritonavir or Indinavir. Furthermore in uninfected subjects only CFU-GM was increased to a significant level with the addition of both PIs, whereas in HIV-1 infected patients BFU-E, CFU-GM and CFU-GEMM were all increased. The weekly addition of PI to BMMC long-term cultures determined a considerable increase in the cellularity, in parallel with an increase in the number of secondary CFCs. No modification on cytokines production has been observed at BM level after the addition of PIs. At baseline and after Ritonavir or Indinavir added, stromal layers resulted positive for CD68 and CD14, but negative for S100 and CD34 molecules, indicating that these cells were of the macrophage/monocyte lineage. In HIV-1 infected patients, the majority of the stromal cells appeared as large and rounded, whereas after the addition of Ritonavir or Indinavir about 60 to 80% of the stromal cells exhibited “fibroblast-like” morphology. Ritonavir and Indinavir increased colony growth of BM obtained either from HIV-1 infected patients and from uninfected individuals, suggesting a clinical role of these drugs in an improvement of haematological parameters also in conditions other than HIV-1 infection. The morphological changes in the stromal cells that we observed may be correlated to the functional changes induced by PIs.
NEW ANTIRETROVIRAL DRUGS

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About fifteen years have elapsed since the early trials of antiretroviral therapy in subjects with AIDS. With seventeen antiretroviral drugs currently available for clinical use and a remarkable number of new agents in the pipeline, we can reasonably expect new hopes for extensively treated patients who have run out of other options.

There are some requirements that any new candidate to clinical use in HIV infection should meet: high potency, activity against viral strains that have become resistant to previous drugs, good tolerability and favorable pharmacokinetics (allowing good compliance by the patients). Only for drugs with such characteristics a durable and successful “life” can be expected.

In general, antiretrovirals can be classified as “new” both if they act through a new mechanism and if they belong to an “old” and known pharmacologic group but show unprecedented and favorable characteristics.

In the first group, the most representative agents include those interfering with extracellular phases of infection (CD4 and co-receptor inhibitors, fusion and penetration inhibitors). T20 (enfuvirtide) is an entry inhibitor that has been recently approved worldwide, and is expected to provide a valuable option to patients who are no longer responding to other therapies. Other agents, such as those inhibiting HIV binding to CD4 and co-receptors, are in a less advanced phase of development.

The enzyme integrase, that is required for the permanent insertion of viral genome into host DNA, is another target for new antiretroviral agents. Integrase inhibitors have shown efficacy in animal models and are currently in phase I-II of clinical development.

In addition to these compounds, very promising drugs in the “old” pharmacologic groups are under investigation. They include inhibitors of reverse transcriptase (both nucleoside analogues and non nucleosidic inhibitors) and protease inhibitors. In general, their advantages include activity against drug resistant HIV strains, low toxicity and favorable pharmacokinetic profile, frequently allowing a once-a-day administration. Emtricitabine and TMC 125 are among the most representative and near-to-come agents in this last group.
Session II
HAART direct effects on agents of opportunistic infections
Chairpersons
Antonio Cassone, Luigi Ortona
SMALL PEPTIDOMIMETICS INHIBITING BOTH HIV-1 AND C. ALBICANS ASPARTIC PROTEASES

Domenico Romeo (a), Alessandro Tossi (a), Damiano Skrbec (a), Fabio Benedetti (b), Federico Berti (b)

(a) Dipartimento di Biochimica, Biofisica e Chimica Macromolecolare, Università di Trieste
(b) Dipartimento di Chimica, Università di Trieste

Since several years, we have contributed to the design and production of efficient transition state peptidomimetic inhibitors of the HIV-1 aspartic protease (HIV-PR). More recently some of these inhibitors have also been tested on Sap2, the secreted aspartic protease of C. albicans, that we have isolated and purified from the culture medium of a virulent strain of the fungus.

We have applied a highly flexible method for rapidly assembling symmetric and asymmetric dihydroxyethylene- and monohydroxyethylene-based peptidomimetics. In the former case, the central, non-cleavable peptide bond isostere was synthesised under stereochemically controlled conditions to yield the $S,S,S,S$ epimer, while in the latter case both the $S,S,S$ or $S,R,S$ epimers were obtained. The residues flanking the central core were chosen from a variety of proteinogenic aminoacids and aromatic carboxylic acids, such as kynurenic acid (Kyn) or phenoxyacetic acid (Poa) and its derivatives. The molecular weights of the synthesised compounds ranged from 553 to 955 Da and their calculated logPo/w ranged from 3.6 to 7.6.

A dozen hexa- or penta-peptidomimetics had IC$_{50}$ towards the HIV-PR lower than 10 nM (under our assay conditions, ritonavir, indinavir and nelfinavir had IC$_{50}$ of 3.0, 1.3, 1.9 nM, respectively). Seven compounds had IC$_{50}$ towards Sap2 lower than or equal to 5 microM (under our assay conditions, ritonavir, indinavir and nelfinavir had IC$_{50}$ of 1.2, 50, 234 microM, respectively). In particular, a dihydroxyethylene-based inhibitor, TS-70, Kyn-Val-Phe-(CH$_2$OH-CH$_2$OH)-Phe-Val-Kyn appears to be the most efficient with an IC$_{50}$ of 0.2 microM.

In general, for the various peptidomimetics there was no correspondence between the capacity to inhibit HIV-PR and Sap2. For the viral protease, the S$_2$ subsite appears to be promiscuous, so that in position P$_2$ of hexameric and pentameric inhibitors Val, Thr or D-2-thienylglycine residues are acceptable and in position P$_2$’ of the pentameric inhibitors dimethyl-Poa is optimal. There does not appear to be a marked difference for $S,S,S$ and $S,R,S$ epimers of the monohydroxyethylene-based inhibitors. For Sap2, Val seemed to be generally the preferred residue in P$_2$ and Poa was preferable to dimethyl-Poa in P$_2$ and/or P$_2$’. Furthermore, for the monohydroxyethylene-based inhibitors, inhibition of Sap2 was higher for the $S,S,S$ epimers. Some of these observations were explained by molecular modelling of complexes between Sap2 and selected inhibitors. The binding site of Sap2 is narrower than that of HIV-PR, and the S$_2$S$_2$’ subsites impose more stringent requirements on residues that can be accepted. For example, the calculated complexation energies were 6.8 kcal/mol less favourable for the $S,R,S$ epimer TS-96, Poa-Phe-(CH$_2$OH-CH$_2$OH)-Phe-Poa, than the $S,S,S$ epimer, while it was 40 kcal/mol less favourable for the $S,S,S$ TS-98, dmpoa-Phe-(CH$_2$OH-CH$_2$OH)-Phe-dmpoa analog.
When *C. albicans* H12 was grown in YCB-BSA medium for 48 hours, in the absence and in the presence of 50 microM TS-70, it was observed that the peptidomimetic inhibitor significantly prevented both the complete hydrolysis of BSA and fungal growth. Growth inhibition, however, did not exceed one logCFU, as even a minimal residual activity of Sap2, or presence of other Saps in the medium, might contribute to *Candida* nutrition and growth.
HAART AND CANDIDIASIS

Antonio Cassone
*Dipartimento Malattie Infettive, Parassitarie ed Immunomediate, Istituto Superiore di Sanità, Roma*

HIV-positive patients under HAART suffer significantly fewer oral infections with the opportunistic fungal pathogen *Candida albicans* than non-HAART-treated patients. One component of HAART is an inhibitor of the HIV protease, the enzyme required for correct processing of retroviral precursor proteins. Several coherent biochemical and microbiological data suggested to us that HIV protease inhibitors might have a direct inhibitory effect on a key virulence factor for mucosal candidiasis, i.e. the secreted aspartic proteinases (Saps). This suggests that the reduction in *C. albicans* infections in HIV-positive patients might not be solely the result of improved immunological status but could also be caused by the HAART treatment directly inhibiting Candida proteinases.

To answer this question, we carried out a controlled, randomised, longitudinal study in which therapy-naïve HIV-positive subjects receiving PI-HAART were matched with subjects under an NNRTI–HAART regimen, all patients being followed up for incidence of oral candidiasis (OC), oral Candida carriage, immunoreconstitution and, importantly, Candida Sap levels in the oral cavity. While confirming that PI-HAART is extremely beneficial against OC, we also noticed that of the 80% Sap-positive subjects at the beginning of PI-HAART, more than half no longer had Sap in their saliva after only two weeks of treatment, and almost all subjects converted to Sap negativity after 30 days of treatment. Conversely, the Sap-positive subjects receiving NNRTI-HAART maintained their positivity after 30 days, and the majority had Sap in the saliva for the whole duration of follow-up (180 days). Importantly, *C. albicans* was not eradicated from the oral cavity, rather its virulence factor was inhibited. Interestingly, the immunoreconstitution of systemic anti- Candida cell-mediated immunity (CMI), although somewhat inconsistent in all subjects, was nonetheless more pronounced in subjects under an NNRTI-HAART regimen than in those receiving PI-HAART. This definitely shows that PIs, but not other HAART drugs, have a specific anti-Sap effect in patients, which correlates with an anti-OC effect and, of course, also correlated with the previously shown anti-candidal effect of PI *in vitro*.

Overall, laboratory and clinical evidence strongly suggest that both immunoreconstitution and anti-Sap effect of PI are responsible for the remarkable anti-Candida curative effects of PI-HAART.
HIV PROTEASE INHIBITORS AND P. CARINII

Chiara Atzori, Antonietta Cargnel
Secondo Dipartimento di Malattie Infettive, Ospedale Luigi Sacco, Milano

Pneumocystis carinii is a major cause of pneumonia in patients with AIDS and in other immunocompromised patients. Since the introduction of HIV PIs, dramatic declines in all OI, including P. carinii pneumonia (PCP), has been noticed among AIDS patients. A recent study reported improved survival with HAART in HIV patients with severe PCP. Explanation for a possible survival benefit from HAART includes decreased viral fitness and an attenuated rise in viral load during PCP but drop in P. carinii events and other OI was observed also during viral failure and before evidence of immune reconstitution in HIV patients, suggesting other alternative mechanisms. We first demonstrated by zymograms the presence of seven cysteine-serine type proteases (molecular weights range: 40-98 kDa) in rat-derived P. carinii trophozoites; other groups then showed a P. carinii proteases multigene family with homology to subtilisin-like serine proteases. Presence of aspartyl proteases in rat-derived P. carinii trophozoites zymograms and anti Pneumocystis dose-dependent activity of Pepstatin A (progenitor of acid proteases) in a well established in vitro model were then described. Rat-derived P. carinii trophozoites from animal model were inoculated onto HEL299 cells maintained in multiwell plates; confluent monolayers were inoculated with rat P. carinii and incubated in the presence of increasing concentrations of several PIs, with demonstration in this model of a partial inhibitory effect exerted by RTV, SQV, IDV, NFV, APV and LPV at concentrations clinically achievable in vivo. The newer HIV PI Atazanavir, also tested against P. carinii, surprisingly increased the amount of actively growing trophozoites in a dose-dependent fashion in repeated experiments. These data seem to indicate that the antipneumocystis activity is not a general effect of the PIs drug-class, and that specific factors led to a completely different impact on in vitro growth. Among PIs, the dose-dependent effect was particularly evident for APV, a sulphamide-like drug. Another group did not confirm our observations in axenic bioluminescent luciferin-luciferase ATP in vitro assay. Interestingly, cresyl violet stain used to score slides, showed in vivo a slight decrease in cysts count in animal lungs treated with PIs; this stain may lead to underscore the degree of infection colouring also empty (dead) cysts, confirming rather conflicting with our in vitro data. Further, differences in results obtained with P. carinii testing PIs in cell-based vs. cell-free systems, suggest an important role of microorganism-host interaction as possible protease-anti protease target. We also recently detected the presence of LPV/r in lung ELF at concentration exceeding those seen as partially inhibitory to P. carinii in vitro. This result could explain the dramatic drop in PCP during the early phase of HAART, prior to immune reconstitution, the improved survival of HIV-infected patients with severe PCP treated with PIs-based HAART being the result of synergistic action of specific and aspecific anti Pneumocystis drugs. It has been demonstrated also that PIs, particularly RTV, appeared to inhibit the chymotrypsin-like activity of host proteasome in vitro suggesting non-viral aspecific modulation in cells. In order to assess differences in immune reconstitution under PI-based versus non-nucleoside reverse transcriptase inhibitor (nNRTI)-based HAART, we studied
lymphocyte proliferation (LP) responses to several antigens in HIV patients. About 30% of immune reconstituted patients (CD4>200) receiving either PI-based or nNRTI-based HAART lacked \textit{P. carinii} specific LP answers, however only among those treated with nNRTI developed PCP with CD4>250 after stopping primary prophylaxis. These data confirm the clinical observation that certain HIV-infected patients retain a relative immunodeficiency that is not reflected in the CD4 cell counts, and they are at risk of developing opportunistic events. Again, a partial, aspecific but clinically protective effect against \textit{P. carinii} could be hypotesized for immune reconstituted patients receiving PIs in their HAART regimens, since no patient with abnormally low Pc-specific LP receiving PIs developed PCP, although prophylaxis was stopped. Several authors reported other antiopportunistic effects either \textit{in vitro} (e.g., \textit{Toxoplasma} and \textit{Candida}) and against Kaposi’s sarcoma. In conclusion, even if aspecific and partial, the antipneumocystic and anti-opportunistic protective effects of most PIs may be clinically relevant \textit{in vivo}. We think that, based on the indirect evidences collected up to date, a PIs-based regimen should be preferably considered to start HAART in heavily immunosuppressed patients with very low CD4 nadir, not excluding a prompt shift to other PIs-sparing regimens as soon as CD4 count reach a safe threshold.
HIV PROTEASE INHIBITORS REDUCE CRYPTOSPORIDIUM PARVUM INFECTION IN BOTH IN VITRO AND IN VIVO MODELS

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Dipartimento Malattie Infettive, Parassitarie ed Immunomediate, Istituto Superiore di Sanita, Roma

In HIV infected persons, Cryptosporidium parvum causes chronic diarrhoea, which can be life-threatening in persons with AIDS and with a low CD4 T cell count. However, a specific and effective therapy for this opportunistic infection does not yet exist. The use of highly active antiretroviral therapy (HAART) in persons with AIDS has reduced the prevalence of infection with C. parvum and the length and severity of its clinical course. This effect has in most cases been attributed to the recovery of the host immunity; however, improvements in this opportunistic infection have been demonstrated even in the absence of immunological recovery, this fact suggests that human HIV-protease inhibitors (HIV-PIs) may be capable of control C. parvum infection. The aim of the present study was to investigate the effect of HIV-PIs on C. parvum infection, since previous papers had demonstrated a direct effect of some HIV PIs on opportunistic infections (Candida, Pneumocystis carinii and Toxoplasma gondii). Among the high number of PIs, we selected the indinavir for our experiments, since a resolution of cryptosporidial enteritis in a person with AIDS after treatment with this drug has been reported. To evaluate the efficacy of indinavir in an in vitro and an in vivo model infected with C. parvum, a method based on the flowcytometric analysis has been developed. In vitro, the treatment of the sporulated oocysts with concentrations of indinavir ranging from 0.1 μM to 50 μM reduced at 24h p.i. the percentage of HCT-8 infected cells in a dose-dependent manner (resulting in a reduction of 46%, 67%, and 100%, respectively at 10, 20 and 50 μM). For established infection, the treatment with 50 μM of indinavir decreased the percentage of infected cells in a time-dependent manner, thus treatment for 96 h resulted in a 57.8% reduction of the percentage of infected cells. In vivo, mice treated with indinavir (24 mg/kg) at the same time they were infected with the oocysts showed a 93% reduction in the number of oocysts present in the entire intestinal contents and a 91% reduction in the number of intracellular parasites in the ileum. For established infection, indinavir treatment for ten days reduced the number of oocysts in the whole intestinal content by about 50% and the number of intracellular parasites in the ileum by about 70%. These data show that indinavir directly interferes with the cycle of C. parvum, resulting in a strong reduction of the infection level. Protease inhibitors could be considered good candidates for the treatment of cryptosporidiosis in immunosuppressed persons.
THE EFFECT OF HIV PROTEASE INHIBITORS
ON C. NEOFORMANS VIRULENCE
AND HOST DEFENSES

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The second most commonly affected organ in AIDS patients is the brain, which is targeted by many opportunistic infections such as toxoplasmosis, cryptococcosis and cytomegalovirus. Although the introduction of highly active antiretroviral therapy (HAART) has decreased the incidence of these infections, they remain a major cause of morbidity and mortality. HAART has particularly improved the outcome for AIDS patients with central nervous system infections, therefore therapy should be considered mandatory in these patients.

HAART could have a dual role in protecting against opportunistic infections. One role could be related to a direct effect on the microorganism by an attenuation of the secretion of virulence factors, such as aspartyl proteinases from Candida albicans. The other role could be attributed to the partial reconstitution of a specific and innate immune response. It is therefore noteworthy the improvements produced by HAART in the innate immune response by limiting the acceleration of apoptosis and decrease of chemotaxis of neutrophils from AIDS patients.

In this study we performed experiments to verify 1) the direct effect of the most common protease inhibitors, ritonavir, indinavir and saquinavir, on virulence factors of C. neoformans; and 2) the functional activity of neutrophils from AIDS patients during HAART.

The results show that these protease inhibitors were able to affect the expression of some virulence factors of C. neoformans. This fungus was less aggressive when injected intravenously into mice in our experimental model. Neutrophils from HIV-infected patients showed altered effector and secretory functions in response to C. neoformans and HAART tends to normalize O2 production, anticytotoxic activity and IL-12 secretion.

In conclusion, beneficial effects of HAART could be explained by the quenching of C. neoformans virulence and by restoring the suppressed functions of neutrophils, thereby ensuring the safe clearance of obligate or facultative intracellular pathogens.
HAART AND CRYPTOCCOCUS: EFFECT OF INDINAVIR ON CRYPTOCCOCUS NEOFORMANS AND ITS PROTEASE(S)

Cristina Mussini (a), Marcello Pinti (b), Elisabetta Blasi (c), Cristian Bellodi (b), Lorenzo Galluzzi (b), Milena Nasi (b), Bruna Colombari (c), Andrea Bedini (a), Andrea Cossarizza (b), Roberto Esposito (a)
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Our group has shown that patients receiving HAART who discontinued prophylactic regimens against C. neoformans before reaching 100 cells/μL did not show recurrences.

Laboratory strains and clinical isolates were exposed to indinavir at different times and different doses and then tested for vitality, mitochondrial activity, replicative capacity, susceptibility to phagocytosis and killing by murine cerebral immune-effectors (BV2 cells) and protease production. A second step was to isolate the protease gene(s) of C. neoformans, that was never done before, and to see the direct effect of indinavir on it.

In vitro, indinavir significantly decreases the replicative activity of C. neoformans. This effect is time-dependent and dose-dependent and similar to what obtained by using pepstatine as positive control (50-70% inhibition at 10-50uM indinavir). Moreover, the pre-exposure of C. neoformans to indinavir increases its susceptibility to microglial cells (200% increase in phagocytosis and killing). As evaluated by azoalbunin hydrolysis assay, protease activity resulted significantly impaired in C. neoformans exposed to indinavir (30-60% decrease with respect to controls). The second part of the study was to isolate the enzyme. Using the aminoacid sequence of SAP5 from C. albicans as a query, we identified an EST clone containing a cDNA from C. neoformans H99 with significant homology for eukaryotic aspartyl proteases. We used this sequence to do a BLAST search of the partially completed C. neoformans genome sequence and we identified the genomic region containing the putative aspartyl protease gene. RACE technology was used to obtain full length cDNA, which was sequenced together with genomic DNA. The gene includes three introns with splice sites perfectly preserved and encodes a putative protein of 505 AA (51.6 kDa). An homology search revealed a significant degree of similarity (35-45%) of CnAP1 to several aspartyl proteases; in particular, essential aminoacids are perfectly conserved. The C-terminal portion of the protein (AA 380-505) does not show any similarity with known proteins and its potential function remains to be analyzed. We have cloned the entire cDNA in pQE80L vector, so adding a 6 His tag at the N terminus of the protein to purify recombinant protein by affinity column. We are now testing the enzymatic activity of the protein in the presence or not of indinavir.

In vitro, indinavir affects the replicative capacity of C. neoformans, its protease production and its susceptibility to microglial cells. A sequence coding for cryptococcal protease has been identified, cloned and the recombinant protein isolated.

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Session III

Other unconventional HAART benefits

Chairpersons
Giancarlo Majori, Mauro Moroni
HAART AND PROTEOSOME

Pier Angelo Tovo
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Not submitted.
HIV-PROTEASE INHIBITORS MODULATE APOPTOTIC PRONENESS OF ACTIVATED T LYMPHOCYTES VIA A TARGET EFFECT ON THEIR MITOCHONDRIA

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Mitochondria are known to generally play a key role in apoptotic cascade. In particular, an alteration or decrease of mitochondrial membrane potential has been hypothesized to be a marker of cells undergoing apoptosis. As for T lymphocytes, T cells have been shown to be susceptible to receptor-mediated apoptosis only once activated. On the other hand, it was recently demonstrated that HIV-protease inhibitors (PIs), independently from any viral infection, can hinder lymphocyte apoptosis by influencing mitochondrial homeostasis. To analyze the mechanisms underlying these effects a specific study was undertaken in both resting and activated human peripheral blood lymphocytes exposed to either receptor- (e.g., anti-Fas) or non-receptor- (e.g., radiation) mediated apoptotic stimuli. T cell activation and apoptotic susceptibility to both types of stimulation were found to be accompanied by a significant increase in mitochondrial membrane potential, or hyperpolarization, which was undetectable in resting cells. We also detected apoptotic hindering exerted by PIs only in activated T lymphocytes. In contrast, resting cells, whose mitochondria were not hyperpolarized, even undergoing apoptosis (i.e. by radiation) remained unaffected by PI exposure. This was apparently due to the ability of these drugs to block activation-associated mitochondria hyperpolarization, which, in turn, was paralleled by an impairment of cell cycle progression. Remarkably, HIV infected cells from naïve patients behaved identically to activated T cells, displaying hyperpolarized mitochondria, while lymphocytes from patients under HAART (which included PIs) seemed to react as resting cells. Finally, PIs also prevented zidovudine (AZT)-mediated mitochondrial toxicity. Altogether these results clearly indicate that the hyperpolarization state of mitochondria may represent a prerequisite for the sensitization of lymphocytes to the so-called activation-induced cell death. They also suggest that HIV protease inhibitors, by interfering with induction of the mitochondrial hyperpolarization state, can result in cell survival even independent of any viral infection.
ANTI-ANGIOGENIC AND ANTI-TUMOR ACTIVITY OF HIV PROTEASE INHIBITORS

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HIV-1 protease inhibitors (HIV-PI) are anti-retroviral drugs that block the HIV aspartyl protease that is required for the production of infectious viral particles. Although HIV-PI have been designed to selectively bind the HIV protease catalytic site, evidence indicates that they can also interfere with several cellular and microbial pathways. In this context, a reduced incidence or regression of Kaposi’s sarcoma (KS) and some types of non Hodgkin lymphomas has been described in HIV-1-infected patients treated with combination regimens containing HIV-PI. We have recently shown that HIV-PI have direct anti-KS and anti-tumour activity, which is due to their capability of blocking both angiogenesis and tumour cell invasion. These effects of HIV-PI occur at the same drug concentrations present in plasma of treated individuals, and are due to the inhibition of the activation of matrix metalloprotease-2, an enzyme that is key to angiogenesis and tumour growth and invasion. The anti-inflammatory activity of HIV-PI, however, may also contribute to the anti-KS effects observed in treated individuals, since it blocks production of cytokines involved in KS initiation and maintenance. Thus, by direct and indirect activities HIV-PI can simultaneously block several pathways involved in cancer growth, invasion or metastasis. These data indicate that HIV-PI should also be investigated and exploited for the therapy of KS and tumours of different histology occurring in both HIV-infected and non-infected individuals. To this goal, a multicentric phase II clinical trial with indinavir in non-HIV-associated KS has just started in Italy.
MODULATOR ACTIVITY OF PIS IN DOXORUBICIN-SELECTED MULTIDRUG RESISTANT KAPOSI’S SARCOMA CELL LINE

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A number of drugs may induce/enhance the multidrug resistance (MDR) phenotype. In human cancer cells, this is often associated with an increased expression of membrane transporters such as P-glycoprotein (P-gp) and the multidrug resistance-associated protein (MRP) that extrude cytotoxic drugs from the cell. Several substances that interact with P-gp and/or MRP and block drug efflux have been reported to reverse the MDR phenotype. P-gp and MRP expression has been investigated in Kaposi’s sarcoma (KS) and KS spindle cells have been found to express both proteins. Moreover, in the case of advanced AIDS-related Kaposi’s sarcoma (AIDS-KS), relapse or progression after prior combination chemotherapy or anthracycline therapy is not uncommon. Therefore, since these transporters might contribute to drug-drug interactions and intracellular concentrations of potentially co-administered drug substrates i.e. antivirals (PIs and NRTIs) and MDR-associated cytotoxic compounds (anthracyclines and Vinca alkaloids), we analyzed this aspect in KS-derived cell lines as well as the inducibility of the MDR phenotype following prolonged exposition to the anthracycline compound doxorubicin. For the development of drug-resistant sublines, the KS-derived SLK cell line was exposed to doxorubicin (DOX) and three resistant sublines designated as SLK-D12, SLK-D25 and SLK-D50 were developed by exposing the parental cells to DOX at concentrations of 5ng/mL to 0.12, 0.25 and 0.50 µg/mL, respectively. P-gp functionality was assessed by rhodamine 123 (Rh123) efflux assay analysed by flow cytometry in the presence or absence of verapamil and the PIs IDV and RTV (1-100µM) while MRP1 function was investigated using carboxyfluorescein (CF). Anti-cancer drug effect on SLK cell proliferation was evaluated using the MTT dye assay. SLK and SKImm cells expressed low levels of P-gp and MRP and efficiently effluxed Rh123 and CF as well as the naturally fluorescent anthracycline compound doxorubicin. A dose-dependent inhibition of P-gp and MRP-mediated efflux was obtained by using PIs. SLK-D sublines were characterized by a proportionally higher expression of P-gp that caused a decrease of Rh123 and DOX accumulation thus requiring higher concentrations of PIs in order to increase the dye retention. Finally, SLK-D sublines appeared to be about 2.5-3.5-fold more resistant to DOX than the parental cell line while PIs increased the sensitivity of resistant cells. Our data demonstrate that KS-derived cell lines may develop an MDR phenotype and that PIs are able to down-modulate P-gp-associated resistance by functioning as MDR-reversing agents. These results may contribute to the positive impact of HAART on KS patients treated with systemic chemotherapy.
HAART AND POVERTY-RELATED DISEASES:
THE SPECIAL CASE OF MALARIA

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Malaria and AIDS are major diseases of mankind, each accounting for 3 millions of deaths per year approximately. The reciprocal negative interactions between these two infections impose preventive and therapeutic action on both. This work relates to the anti-HIV-1 effects of chloroquine (CQ) and the direct antimalarial effects of the inhibitors of the HIV-1 aspartic protease (PIs) and to the combined capacity of PIs plus CQ of inhibiting both HIV-1 and Plasmodium sp.

First, we evaluated the anti-HIV-1 effects of CQ in association with PIs. In combination with indinavir, ritonavir or saquinavir, CQ had a synergistic effect at concentrations found in plasma of subjects under antimalarial prophylaxis. Of note, CQ restored the response to indinavir in a multidrug-resistant primary isolate. Moreover, CQ increased the block of P-gp and MRP-mediated efflux exerted by PIs in CD4+ lymphocytes.

Then, we estimated the antiplasmodial effects of PIs, alone or in combination with known antimalarials, based on the consideration that, in P. falciparum, four enzymes, i.e. the plasmepsins, have aspartic-protease activity. Using the VAST algorithm, we found that plasmepsins share significant structural and sequence homologies with the HIV-1 protease. Accordingly, we found that indinavir, ritonavir and saquinavir dose-dependently inhibited the aspartic-protease activity of a lysate of P. falciparum trophozoites. In human P. falciparum-parasited erythrocytes treated with concentrations of PIs reachable in plasma of individuals under PI-therapy, we found that indinavir, ritonavir and saquinavir dose-dependently inhibited P. falciparum growth, independently of its CQ-resistance profile. In assays using CQ plus a PI at concentrations sub-optimally inhibiting P. falciparum growth, we found that the effects of the CQ/PI combination were synergistic in drug-resistant P. falciparum and additive in drug-sensitive parasites. We conclude that PIs restore sensitivity to CQ. Finally, in preliminary tests, ritonavir exerted antimalarial effects in mice inoculated with P. berghei.

The ability of PIs to partially restore chloroquine sensitivity in P. falciparum is strikingly similar to their ability to rescue drug sensitivity in mammalian cancer cells with a multidrug resistant phenotype. We are currently investigating the possibility of a combined block of P-glycoprotein and MRP1 behind the synergistic antiplasmodial and anti-HIV-1 effects of the CQ/PI combination.

If these observations are confirmed in clinical trials, they will open a scenario wherein the antimalarial and anti-HIV synergism of PIs and CQ may help fight the synergism between two global killers, malaria and AIDS.
## AUTHORS’ INDEX

<table>
<thead>
<tr>
<th>Author</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aiuti, F.; 6</td>
<td></td>
</tr>
<tr>
<td>Atzori, C.; 14</td>
<td></td>
</tr>
<tr>
<td>Autran, B.; 5</td>
<td></td>
</tr>
<tr>
<td>Bedini, A.; 18</td>
<td></td>
</tr>
<tr>
<td>Bellodi, C.; 18</td>
<td></td>
</tr>
<tr>
<td>Benedetti, F.; 11</td>
<td></td>
</tr>
<tr>
<td>Berti, F.; 11</td>
<td></td>
</tr>
<tr>
<td>Blasi, E.; 18</td>
<td></td>
</tr>
<tr>
<td>Cargnel, A.; 14</td>
<td></td>
</tr>
<tr>
<td>Cassone, A.; 13; 22</td>
<td></td>
</tr>
<tr>
<td>Cauda, R.; 22</td>
<td></td>
</tr>
<tr>
<td>Colombari, B.; 18</td>
<td></td>
</tr>
<tr>
<td>Cossarizza, A.; 18</td>
<td></td>
</tr>
<tr>
<td>Ensoli, B.; 23</td>
<td></td>
</tr>
<tr>
<td>Esposito, R.; 18</td>
<td></td>
</tr>
<tr>
<td>Galluzzi, L.; 18</td>
<td></td>
</tr>
<tr>
<td>Gambardella, L.; 22</td>
<td></td>
</tr>
<tr>
<td>Gomez Morales, M.A.; 16</td>
<td></td>
</tr>
<tr>
<td>Isgrò, A.; 6</td>
<td></td>
</tr>
<tr>
<td>Lucia, M.B.; 24</td>
<td></td>
</tr>
<tr>
<td>Malorni, W.; 22</td>
<td></td>
</tr>
<tr>
<td>Matarrese, P.; 22</td>
<td></td>
</tr>
<tr>
<td>Mele, R.; 16</td>
<td></td>
</tr>
<tr>
<td>Mussini, C.; 18</td>
<td></td>
</tr>
<tr>
<td>Nasi, M.; 18</td>
<td></td>
</tr>
<tr>
<td>Pinti, M.; 18</td>
<td></td>
</tr>
<tr>
<td>Pozio, E.; 16</td>
<td></td>
</tr>
<tr>
<td>Romeo, D.; 11</td>
<td></td>
</tr>
<tr>
<td>Savarino, A.; 25</td>
<td></td>
</tr>
<tr>
<td>Skrbec, D.; 11</td>
<td></td>
</tr>
<tr>
<td>Tosini, F.; 16</td>
<td></td>
</tr>
<tr>
<td>Tossi, A.; 11</td>
<td></td>
</tr>
<tr>
<td>Tovo, P.A.; 21</td>
<td></td>
</tr>
<tr>
<td>Vecchiarelli, A.; 17</td>
<td></td>
</tr>
<tr>
<td>Vella, S.; 7; 22</td>
<td></td>
</tr>
<tr>
<td>Weller, I.; 1</td>
<td></td>
</tr>
</tbody>
</table>
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