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Effects of NO-Hybridization on the Immunomodulatory Properties of the HIV Protease Inhibitors Lopinavir and Ritonavir

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Abstract: HIV protease inhibitors (PIs) are antiretroviral agents, which have been found to also affect several cellular processes, such as inflammation and cell progression. In studies on non-steroidal, anti-inflammatory drugs, the addition of a nitric oxide (NO) moiety has been shown to both reduce their toxicity and enhance their pharmacological efficacy. Along this line of research, several derivatives of PIs have been synthesized by covalent attachment of NO moiety to the parental molecules. Previous work has indicated that NO-hybridization of the prototypical PI, Saquinavir leads to a derivative named Saquinavir-NO that while retaining the antiretroviral effect, acquires antitumoural and immunomodulatory properties along with reduced toxicity *in vitro* and *in vivo*. These data prompted us to evaluate the effects of NO-hybridization on two other PIs, Lopinavir and Ritonavir. The two NO-derivatives were compared head to head with their parental compounds on human primary peripheral blood mononuclear cells as well as on human primary macrophages. Lopinavir-NO and Lopinavir were also screened in an *in vivo* model of autoimmune hepatitis. Our results prove that Lopinavir-NO exerts markedly superior effects as compared to the parental compound both *in vitro* and *in vivo*. On the contrary, Ritonavir-NO effects overlapped those of Ritonavir. These data demonstrate that NO-hybridization of Lopinavir generates a derivative with significantly stronger immunomodulatory effects that are apparently related to an action of the compound on T-cell secretory capacity. Lopinavir-NO deserves additional studies for its possible use in T-cell-mediated autoimmune diseases including, but not limited to autoimmune hepatitis.

Antiretroviral protease inhibitors (PIs) in combination with nucleoside or non-nucleoside reverse transcriptase inhibitors are the basis for the highly active antiretroviral therapy (HAART) that is efficient in suppression of HIV replication and reduction of clinical manifestations of the disease [1].

In addition to their primary pharmacological indication, these drugs have also been described to possess antineoplastic and immunomodulatory properties both in *in vitro* and *in vivo* pre-clinical settings [2–4].

However, serious side effects including dyslipidaemia, insulin resistance, lipodystrophy, hepatotoxicity and atherosclerotic cardiovascular complications [5–9] have dampened the possible extension of these drugs to therapeutic areas different than HIV infection.

It has been previously demonstrated that nitric oxide (NO)-hybridization may reduce toxicity of parental compounds while enhancing the pharmacological potency of the drugs [10]. Along this line of research, several derivatives of PIs have been synthesized at OncoNOx (Copenhagen, Denmark) by covalent attachment of NO moiety to the parental molecules.

Previous work carried out by ourselves and others has indicated that NO-hybridization of the prototypical PI, Saquinavir leads to a derivative named Saquinavir-NO that while retaining the antiretroviral effect acquires antitumoural [11–17] and immunomodulatory [12,18,19] properties along with reduced toxicity *in vitro* and *in vivo* [12].

These data prompted us to evaluate the effects of NO-hybridization on two other PIs that have been reported to possess immunomodulatory properties, such as Lopinavir and Ritonavir [3,4]. The two NO-derivatives were compared head to head with their parental compounds on human primary peripheral blood mononuclear cells as well as on human primary macrophages. Lopinavir-NO and Lopinavir were also screened in an *in vivo* model of autoimmune hepatitis induced by ConA. Our results prove that Lopinavir-NO exerts markedly superior effects as compared to the parental compound both in the *in vitro* and *in vivo* settings. On the contrary, the effects of Ritonavir-NO overlapped those of Ritonavir. These data indicate that NO-hybridization can, but not mandatorily, modify the pharmacodynamics of a compound and candidates Lopinavir-NO as a novel potentially potent and important immunomodulatory drug.

Material and Methods

Reagents. RPMI-1640 medium and foetal calf serum (FCS) were from Lonza (Italy). Dimethyl sulfoxide (DMSO) was from Sigma-Aldrich (St. Louis, MO, USA). Lopinavir and Ritonavir were

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purchased from Hoffman-La Roche. Lopinavir-NO and Ritonavir-NO were obtained from OncoNox (Copenhagen, Denmark). ConcanavalinA (ConA) and lipopolysaccharides (LPS) from *Salmonella enterica* were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Synthesis of Lopinavir-NO and Ritonavir-NO. Lopinavir (0.24 mmol) and Ritonavir (0.35 mmol) in $CHCl_3$ were added to a stirring mixture of fuming nitric acid ($\geq$ 90% HNO_3) and Ac_2O at -20°C for 24 hr under nitrogen. The reaction mixture was quenched with ice-cold water and extracted with CH_2Cl_2. The extracts were washed with ice-cold saturated $NaHCO_3$ and water, dried with $MgSO_4$, and filtered. The solvent was evaporated under pressure, and the crude product was purified by flash column chromatography (FCC) eluting with 4:3:1 hexane: ethyl acetate: methanol. The product obtained was recrystallized from EtOAc/Hexane to give Lopinavir-ONO_2 and Ritonavir-ONO_2 a white solid, and the purity was analysed by HPLC and MS.

Animals. Six- to 8-week-old female CD1 mice were purchased from Harlan-Nossan (San Pietro al Natisone, Udine, Italy). The mice were kept under standard laboratory conditions (non-specific pathogen free) with free access to food and water. The animals used in the experiments were protected in accordance with Directive 86/609/EEC. The animal studies were carried out in accordance with local guidelines and approved by the local Institutional Animal Care and Use Committee (IACUC) (Protocol Number 144/2011).

Hepatitis induction and ALT determination. ConA was dissolved in sterile PBS and injected into the tail veins of mice in a final volume of 100 µl at the dose of 20 mg/kg body-weight. Animals were treated intraperitoneally with either Lopinavir or Lopinavir-NO 10 mg/kg or with its vehicle, 24 hr and 1 hr before the ConA challenge. Animals were killed for blood collection 8 hr after ConA challenge. Mice dead before being killed were not included in the serological analysis. Serum ALT levels were determined using a Reflotron Analyser (Roche) and Reflotron ALT test strips according to the manufacturer's instructions (Roche). Data originate from two independent experiments entailing a total of 10 mice per group. As the data between the different experiments were highly reproducible, the two experiments have been merged and shown as single study.

Cells. Human peripheral blood mononuclear cells (PBMCs) and monocyte-derived macrophages were isolated from the fresh buffy coats of healthy volunteers. The buffy coats were diluted with phosphate-buffered saline (PBS) and layered onto Ficoll–Hypaque gradients (Gibco, Invitrogen, Milan, Italy). After centrifugation, the mononuclear cells at the interphase were collected and washed twice with PBS. PBMCs were cultured in complete RPMI medium (10% FBS, 2 mM glutamine and 50 mg/ml penicillin/streptomycin). For isolation of the monocytes, cells were cultured in Iscove's medium supplemented with 2 mM glutamine and 50 mg/ml of penicillin/streptomycin. After 2-hr incubations, the nonadherent cells were washed out using PBS. The monocytes were then cultured for 7 days in Iscove's medium supplemented with 10% foetal bovine serum (FBS), 2 mM glutamine and 1% of penicillin/streptomycin (Invitrogen, Milan, Italy) to obtain macrophages.

Cell culture and treatment. Lopinavir-NO, Ritonavir-NO and the parental PIs were dissolved in DMSO at the concentration of 100 mg/ml.

PBMCs were seeded at a 2×10^6/ml in 24-well plates in the presence of complete medium or ConA 5 µg/ml for 24 and 48 hr and in the presence or absence of test compounds at the final concentration of 1 and 10 µg/ml. At the end of the incubation period, supernatants were collected and stored at -20°C for subsequent determination of cytokines.

Macrophages were cultured in the presence of LPS 10 µg/ml or medium, and of test compounds at the final concentration of 10 µg/ml for 18 hr, after which cells were collected for subsequent RNA extraction.

RNA isolation and real-time RT-PCR. Total RNA was extracted using TRIzol reagent following the manufacturer's instructions (Life Technologies, Monza, Italy). Two micrograms of total RNA was retro-transcribed using the Transcriptor First-strand cDNA Synthesis Kit (Roche, Monza, Italy), and cDNA was used for the determination of cytokines and chemokines by real-time RT-PCR using the FastStart SYBR Green Master (Roche, Monza, Italy). Primer sequences were as follows: TNF-α forward: AAGCACACTGGTTTCCACACT; TNF-α reverse: TGGGTCCCTGCATATCCGTT; interleukin-6 (IL-6) forward: ACTCACCTCTTCAGAACGAATTG; IL6 reverse: CGCAGCTCTAG GAGCATGTG; CXCL2 forward: TGCAGAGAGAAGGGAATCTCG; CXCL2 reverse: ATGTAAGTCTCGCCCATCGC; CXCL10 forward: GTGGCATTCAAGGAGTACCTC; CXCL10 reverse: TGATGGC CTTCGATTCTGGATT; glyceraldehyde 3-phosphate dehydrogenase (GAPDH) forward: TGTGGGCATCAATGGATTTGG; GAPDH reverse: ACACCATGTATTCCGGGTCAAT. Gene expression was calculated using the formula:

$$2^{-\Delta\Delta C_t}, \text{where } \Delta\Delta C_t = [(C_t, \text{target gene} - C_t, \text{GAPDH}) \text{ treated sample} - (C_t, \text{target gene} - C_t, \text{GAPDH}) \text{ control sample}]$$

ELISA. Cytokine concentration in cell culture supernatants was determined by sandwich ELISA using MaxiSorp plates (Nunc, Rochild, Denmark) and anticytokine paired antibodies according to the manufacturer's instructions. Samples were analysed in duplicate for human TNF-α, human IL-10, human IL-6 and human IFN-γ (eBioscience, San Diego, CA, USA). The results were calculated using standard curves made on the basis of known concentrations of the appropriate recombinant cytokines.

Western blot analysis. Cells were stimulated with LPS 10 µg/ml and treated with 10 µg/ml of Lopinavir-NO and equal dose of Lopinavir for 2 hr and lysed in protein lysis buffer containing 62.5 mM Tris-HCl (pH 6.8 at 25°C), 2% (w/v) SDS, 10% glycerol, 50 mM dithiothreitol and 0.01% (w/v) bromphenol blue. Samples were then electrophoretically separated on 12% SDS–polyacrylamide gels. Electrotransfer to polyvinylidenedifluoride membranes at 5 mA/cm^2 was performed using a semi-dry blotting system (Fastblot B43; Bio-Rad, Goettingen, Germany). The membranes were blocked with 5% (w/v) BSA in PBS with 0.1% Tween 20, and then, blots were incubated with specific antibodies to p-p70S6K (Thr421/Ser424), and β-actin (Cell Signaling Technology, Danvers, MA, USA), after incubation with secondary horseradish peroxidase-conjugated antibody. Bands were visualized using a TMB colorimetric system (Sigma).

Viability assay. Cell viability was evaluated using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) reduction assay. Cells (5×10^5 cells/well) were seeded in 96-well plates and treated with increasing concentrations of Lopinavir and Lopinavir-NO. After drug exposure for 24 hr, 10 µl of 5% MTT was added to each well and incubated at 37°C in a 5% CO_2 atmosphere. After careful removal of the supernatant, 100 µl of dimethyl sulfoxide (DMSO) was added to each well and the cells were incubated for 3 hr. After slight shaking for 10 min., the absorbance values of the wells were measured with a 570-/630-nm bandpass filter. The average values were determined from triplicate readings.

Statistical analysis. Data are presented as Mean±S.D. of three independent experiments (two experiments for the *in vivo* data). Normality was checked using the Shapiro–Wilk normality test. Outliers were detected using the Rosner's Extreme Studentized Deviate test and excluded from the analysis. Statistical analysis for significant differences was performed according to Student's *t*-test for unpaired data. Statistical differences in survival were calculated using the log-rank (Mantel–Cox) Test. A value of $p < 0.05$ was considered to be statistically significant.

Results

Effect of Lopinavir-NO on ConA-activated human PBMCs. Lopinavir-NO was synthesized as described in Materials and Methods and analysed via mass spectrometry (fig. 1A) and HPLC (fig. 1B).

To evaluate the comparative effects of Lopinavir and Lopinavir-NO on cytokine production from human PBMCs upon ConA stimulation, cells were incubated for 24 and 48 hr with 1 and 10 μg/ml of test compounds and supernatant collected for subsequent ELISA. The concentrations of drugs were at least 10 times lower than the LD50 concentration of drugs, as evaluated in a cell viability assay on unstimulated PBMCs (fig. 1C). As expected, ConA incubation was associated with a significant increase in the production of IFN-γ, TNF-α, IL-6 and IL-10 at both 24 and 48 hr (fig. 2). Neither Lopinavir nor Lopinavir-NO influenced the secretion of these cytokines from unstimulated PBMC (fig. 2). No significant effects on cytokine secretion were observed for Lopinavir or Lopinavir-NO at 1 μg/ml concentration upon ConA challenge (fig. 2). On

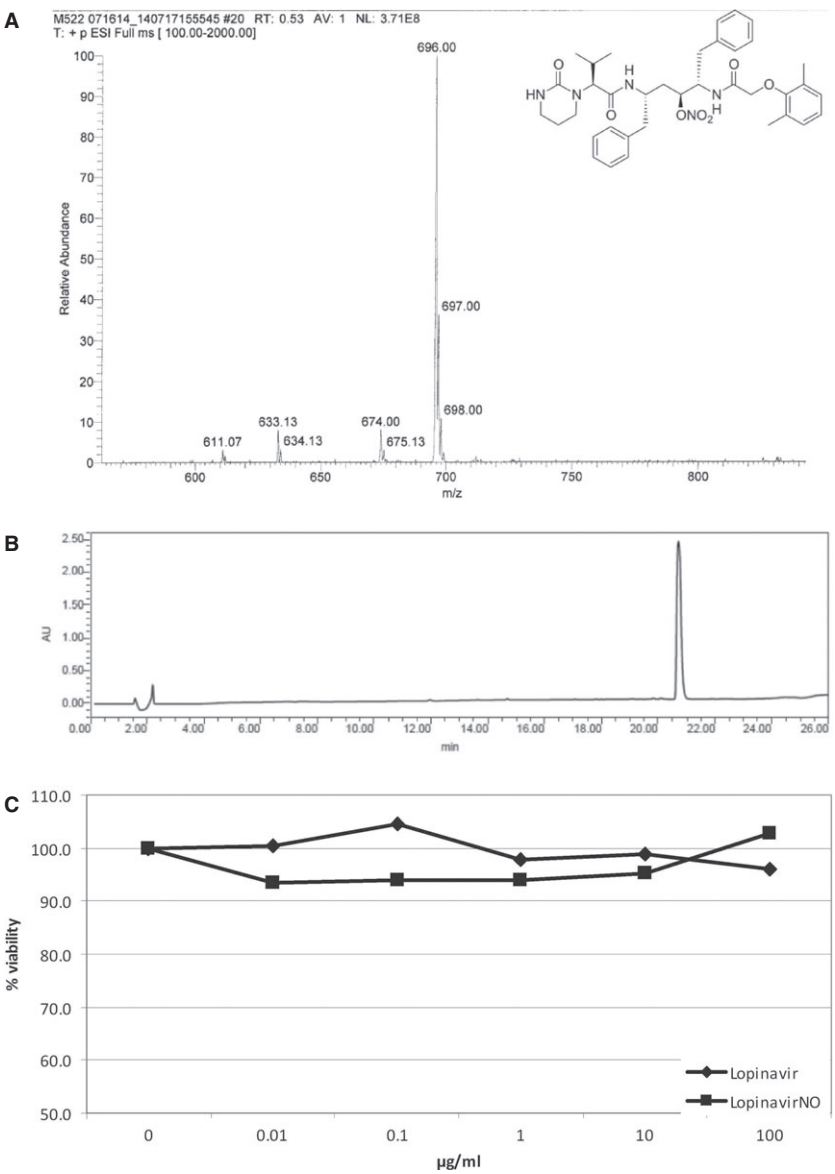

Fig. 1. Characterization of Lopinavir-NO. Low- and high-resolution mass spectrometry was carried out at the LCQ DECA XP MS (A). HPLC spectra obtained at 214 nm using Column Phenomenex primesphere 5 C18 MC 110A 250 × 4.6 mm (B). Human PBMCs were incubated for 24 hr with scalar concentrations of Lopinavir-NO or the parental compound Lopinavir and viability measured by MTT assay. Data are shown as % viability with respect to vehicle, arbitrarily set as 100. Results are from three independent experiments (C).

the other hand, a significant inhibition of ConA-induced secretion of TNF-α and IFN-γ was observed after 24 but not 48 hr of culture when Lopinavir was added at the concentration of 10 μg/ml. The impact of Lopinavir-NO was more powerful and prolonged than that of Lopinavir entailing a significantly lower secretion of TNF-α, IFN-γ that retained the significance at 48 hr of culture. The effect of Lopinavir-NO was also evident for IL-6 and IL-10 that were not influenced from Lopinavir (fig. 2).

In vitro *effects of Lopinavir-NO on human primary macrophages.*

Human macrophages were differentiated *in vitro* from monocytes isolated from the buffy coats of healthy donors. The effects of Lopinavir and Lopinavir-NO were assessed upon LPS challenge in supernatants and total RNA. As previously reported [4], Lopinavir incubation was associated with an induction of the cytokines and chemokines tested, although reaching the statistical significance only for CXCL10. This effect was also observed with Lopinavir-NO but to a smaller extent (fig. 3). Both Lopinavir and Lopinavir-NO were able to reduce TNF-α and IL-6 levels upon LPS challenge (fig. 3A). A significant inhibition of LPS-induced CXCL2 expression was observed for both Lopinavir and Lopinavir-NO (fig. 3B), while an increase in spontaneous and LPS-induced CXCL10 expression was observed for both compounds.

The effects of Lopinavir-NO are independent from the mTOR pathway.

Previous data have shown that the NO-modified Saquinavir is able to significantly modulate the activation of mTOR by inhibiting upstream molecules of the pathway [18]. We wanted to verify whether such property was maintained with Lopinavir-NO. No significant alteration in the level of phosphorylation of p70S6K was observed upon Lopinavir-NO incubation. The actions of Lopinavir-NO are thus different from those of Saquinavir-NO and independent from mTOR (fig. 4).

Lopinavir-NO ameliorates clinical signs of ConA-induced hepatitis.

The *in vitro* data prompted us to evaluate the *in vivo* effects of Lopinavir-NO *versus* Lopinavir in the model of immunoinflammatory hepatitis that can be induced in mice by a single injection with ConA. As we described elsewhere, serological and histological signs of hepatitis can be observed in mice within 8 hr after ConA injection that are characterized by elevation of transaminases activity and inflammatory infiltration of the liver by neutrophils, macrophages and T cells. A variable degree of mortality has also been reported to occur during ConA-induced hepatitis [20]. In this study, ConA administration was associated with 50% death in the vehicle-treated mice, within 8 hr after challenge (fig. 5A). Treatment with

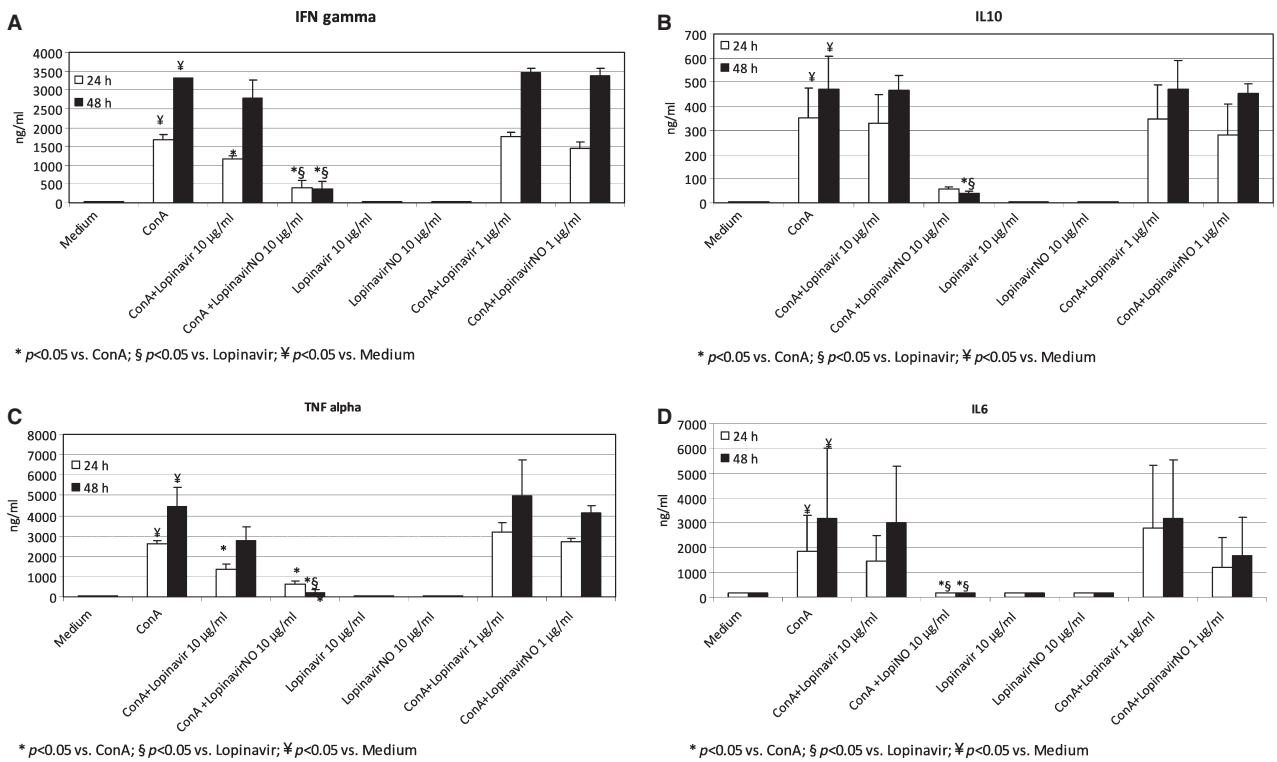

Fig. 2. Effects of Lopinavir and Lopinavir-NO on cytokine production from human PBMCs. PBMCs were isolated from buffy coat of healthy donors and cultured in complete medium in the presence of ConA (5 μg/ml) and Lopinavir or Lopinavir-NO (1 and 10 μg/ml) for 24 and 48 hr. At the end of the treatment period, supernatants were collected for the subsequent determination of IFN-γ (A), IL-10 (B), TNF-α (C) and IL-6 (D) using commercially available ELISA kits. Results are from three independent experiments.

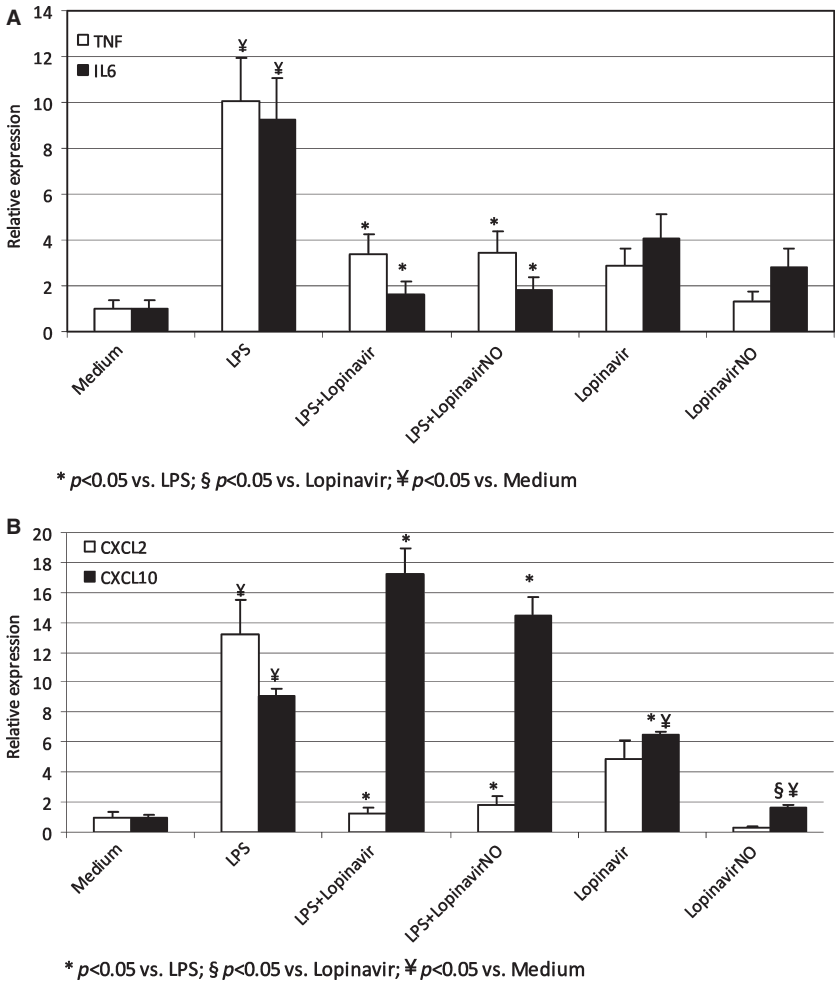

Fig. 3. Comparative effects of Lopinavir and Lopinavir-NO on LPS-stimulated macrophages. Macrophages were obtained from PBMCs of healthy individuals and were stimulated with LPS (10 μg/ml) for 18 hr in the presence of either Lopinavir or Lopinavir-NO for the subsequent extraction of total RNA. Data are from real-time RT-PCR for TNF-α and IL-6 (A) and CXCL2 and CXCl10 (B). Results are from three independent experiments.

Lopinavir-NO completely protected the mice from ConA-induced lethality ($p = 0.01$). As expected, a marked increase in ALT levels was observed in control mice treated with vehicle, 8 hr after ConA challenge (fig. 5B). In contrast to the parental compound, administration of Lopinavir-NO was associated with a significant reduction in the levels of circulating ALT (fig. 5B).

Effect of Ritonavir-NO on ConA-activated human PBMCs.

The encouraging results obtained with Lopinavir-NO led us to evaluate the comparative immunomodulatory properties of Ritonavir-NO and its parental compound Ritonavir under the same experimental conditions used for the comparison of Lopinavir and Lopinavir-NO. A slight induction of IL-10 in unchallenged cells was observed for both Ritonavir and Ritonavir-NO (fig. 6).

Neither Ritonavir nor Ritonavir-NO were able at 1 μg/ml concentration to modulate the secretion of IFN-γ, IL-10, IL-6 and TNF-α levels in the supernatant of human PBMCs stimulated with ConA. A significant reduction, however, was observed at 10 μg/ml for all the cytokine tested, but the effects of the two drugs were superimposable (fig. 6).

In vitro *effects of Ritonavir-NO on human primary macrophages.*

The effects of Ritonavir and Ritonavir-NO were determined on primary human macrophages alone or in association with LPS stimulation. Both compounds increased TNF-α, IL-6, CXCL2 and CXCL10 levels in LPS-stimulated macrophages (fig. 7). A significant reduction of TNF-α and CXCL10 was observed for both Ritonavir and Ritonavir-NO (fig. 7). A trend towards reduced expression was observed for IL-6 and CXCL2 for Ritonavir and Ritonavir-NO (fig. 7). No significant differences were observed between Ritonavir-NO and the parental compound Ritonavir.

Discussion

HIV protease inhibitors (PIs) are antiretroviral agents, approved for human use, designed to selectively bind the

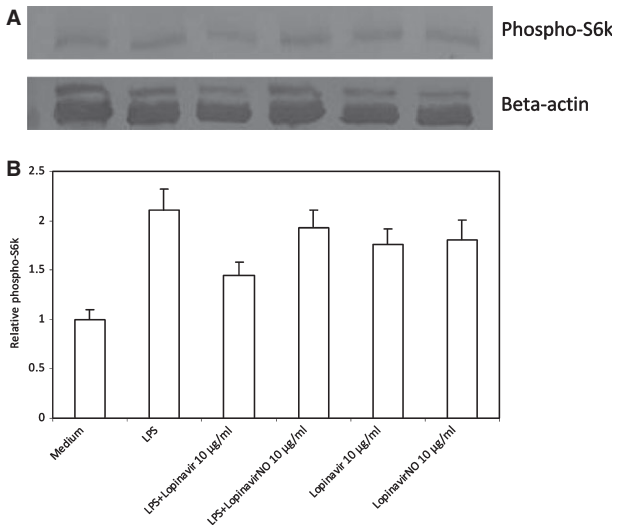

Fig. 4. Western Blot analysis for phospho-S6 kinase on LPS-stimulated PBMCs. PBMCs were stimulated with LPS 10 µg/ml and treated with 10 µg/ml of Lopinavir or Lopinavir-NO for 2 hr and protein extracted for the evaluation by Western Blot of the levels of phospho-S6 kinase. Representative blot (A) and densitometric analysis (B) are shown. Results are from three independent experiments.

catalytic site of the HIV protease, thus blocking the production of infectious virions [21]. It was, however, found that these compounds also affect cellular processes such as angiogenesis, inflammation, the antigen processing and presentation process, cell survival and cell-cycle progression and tissue remodelling [3,22–24]. Data suggest that targets of these drugs may include the PI3K/Akt signalling pathway, nuclear factor κB, signal transducers and activators of transcription 3 (STAT3), matrix metalloproteinase, fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) [25–30].

In studies on non-steroidal anti-inflammatory drugs, the addition of a nitric oxide (NO) moiety has been shown to both reduce their toxicity and enhance their pharmacological efficacy [31]. Along this, previous comparative studies on the immunomodulation exerted by Saquinavir-NO and Saquinavir demonstrated that unlike its parental compound, Saquinavir-NO inhibited the generation of various cytokines in ConA-stimulated unfractionated murine spleen cells and rat lymph nodes stimulated with ConA, as well as in purified CD4(+) T cells *in vitro*, and it also reduced the circulating levels of cytokines in mice challenged with anti-CD3 antibody. It was also observed that Saquinavir-NO, unlike Saquinavir, potently decreased interleukin IL-10, IL-6 and nitrite accumulation and increased the levels of IL-1β and TNF-α in supernatants of mouse and rat macrophage cultures *in vitro* and reduced the blood levels of IL-6 in LPS-treated mice [19]. Furthermore, Saquinavir-NO reduced IL-17 and IFN-γ production in myelin basic protein (MBP)-specific cells isolated from rats immunized with MBP. The effect was associated with inhibition of the phosphorylation of S6 kinases [18]. These findings translated well into the *in vivo* setting as Saquinavir-NO ameliorated the course of the disease in two pre-clinical models of multiple sclerosis.

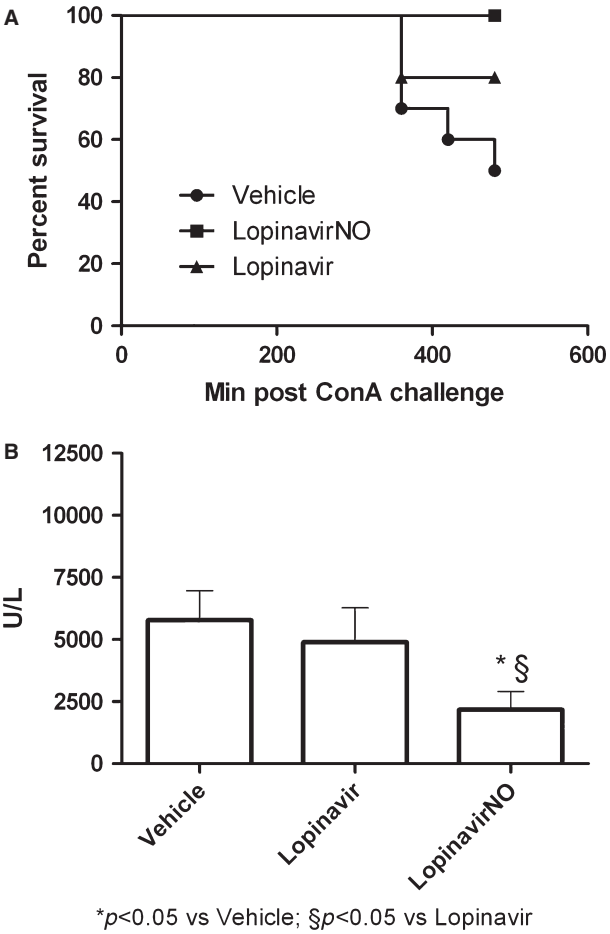

Fig. 5. Effect of Lopinavir and Lopinavir-NO in a model of murine of autoimmune hepatitis. ConA was dissolved in sterile PBS and injected into the tail veins of CD1 mice at the dose of 20 mg/kg body-weight. Animals were treated intraperitoneally with either Lopinavir or Lopinavir-NO 10 mg/kg or with its vehicle, 24 hr and 1 hr before the ConA challenge. Animals were killed for blood collection 8 hr after ConA challenge. Survival curve (A) and plasma ALT levels at time of killing (B) are shown. Data are from two independent experiments merged together (number of mice per group=10).

Our present results indicate that both Lopinavir and Ritonavir at concentrations reached in treated patients [32] are able to modulate the cytokine production by PBMCs and macrophages of healthy individuals. We have also shown that the NO-hybridization significantly alters the immunopharmacological profile of Lopinavir but not that of Ritonavir. Accordingly, Lopinavir-NO exerts more potent immunomodulatory effects than the parental compound Lopinavir. The inhibitory action of Lopinavir-NO on cytokine production as compared to Lopinavir is particularly pronounced on ConA-stimulated PBMCs and results in profound and prolonged suppression of the secretion of IL-6, IL-10, TNF-α and IFN-γ. On the other hand, the NO-hybridization did not influence the impact of Lopinavir on IL-6 and TNF-α secretion from human macrophages as the two compounds exhibited a superimposable action on the spontaneous and LPS-induced secretion of these cytokines. Lopinavir and Lopinavir-NO also exhibited

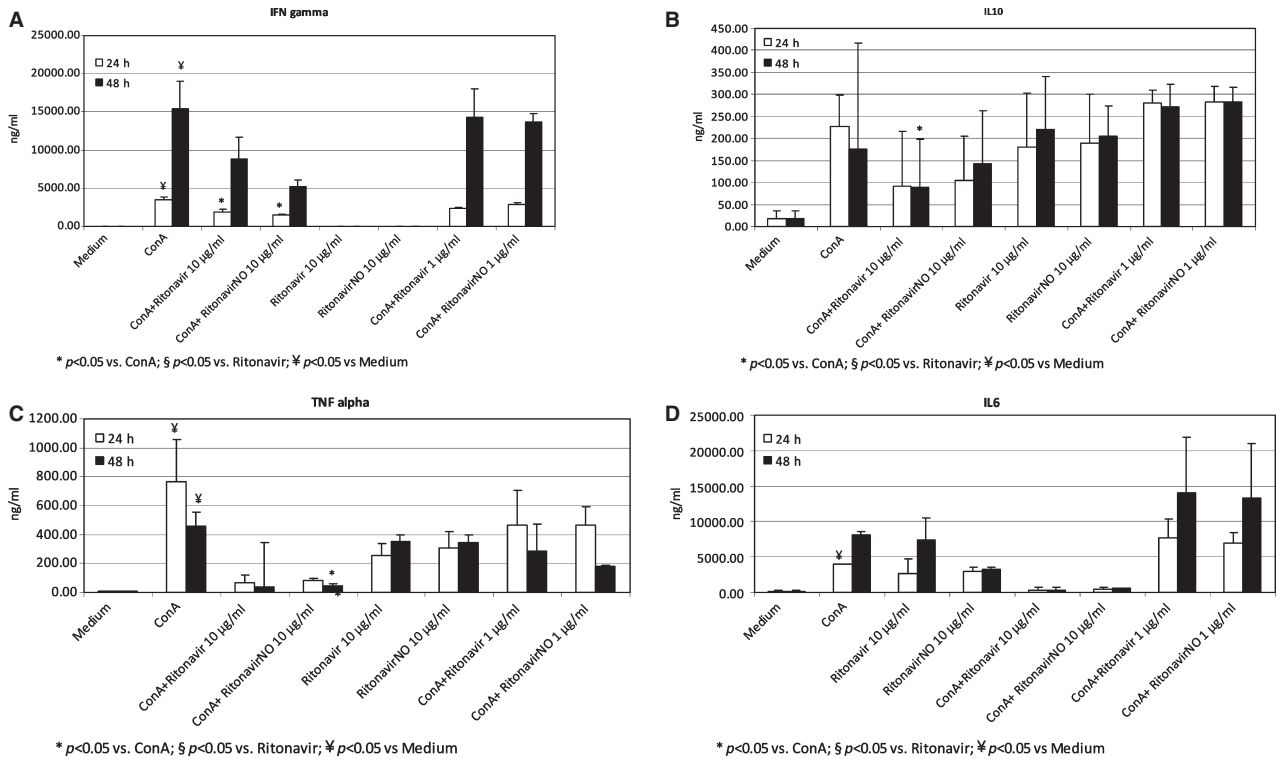

Fig. 6. Effects of Ritonavir and Ritonavir-NO on cytokine production from human PBMCs. PBMCs were isolated from buffy coat of healthy donors and cultured in complete medium in the presence of ConA (5 µg/ml) and Ritonavir or Ritonavir-NO (1 and 10 µg/ml) for 24 and 48 hr. At the end of the treatment period, supernatants were collected for the subsequent determination of IFN-γ (A), IL10 (B), TNF-α (C) and IL-6 (D) using commercially available ELISA kits. Results are from three independent experiments.

comparable effects on the LPS-induced inhibition of CXCL2 and on the increase in spontaneous and LPS-induced expression of CXCL10. Although these data suggest that the T cell may represent the primary cellular target of the immunopharmacological action of Lopinavir-NO, we failed to demonstrate the molecular pathway tackled from the compound in these cells as, in a manner similar to the parental drug, Lopinavir-NO did not influence the phosphorylation of S6 kinase that is the signalling pathway down-regulated from Saquinavir-NO in CD4 T cells [18]. It is noteworthy for the potential translation of these findings that prove a strong and selective action of Lopinavir-NO on cytokine secretion that the inhibitory action occurred at concentrations well below those required to exert toxic effects on human PBMC and that the *in vitro* effect on ConA-induced cytokine secretion was associated with clinical and serological protection in an *in vivo* model of cytokine-dependent liver injury that can be induced in mice by a single injection of ConA. In this model, that is dependent on the co-ordinated action of both type 1 and type 2 cytokines, Lopinavir-NO completely prevented lethality induced by ConA challenge and also led to significant reduction of transaminases levels that witness acute liver damage.

On the contrary, no significant alteration in the properties of the parental molecule was obtained by NO-hybridization of Ritonavir. The effects of Ritonavir-NO were superimposable to those of the parental compound Ritonavir, on both PBMCs and macrophages. These observations indicate that the hybridization of molecule with NO does not '*per se*' affect the immunopharmacological profile of the parental compound.

There are numerous reports on the effect of PIs on cytokine generation in immune cells, although often with discordant results. In an *ex vivo* study on splenocytes obtained from mice treated with Saquinavir produced more IFN-γ and IL-2, less TGF-β and comparable amounts of IL-1β, IL-10 and TNF than cells from control animals [33]. Moreover, HIV patients treated with Saquinavir and Ritonavir showed *ex vivo*-increased production of IL-2, IL-10, IL-12 and MIP-1 in stimulated PBMCs [34,35]. Saquinavir was also shown to induce IFN-γ production in PBMC *in vitro* [36]. However, in other studies, Saquinavir applied as a part of combined anti-HIV therapy *in vivo* or as a single treatment *in vitro*, reduced the percentage of TNF, IL-2 and IFN-γ expressing cells in human unfractionated PBMCs or sorted CD4+ T cells [3,37]. Other reports [38,39] show that Lopinavir is able to induce both TNF-α and IL-6 expression in a time-dependent manner in macrophages, an effect dependent on ERK activation and consequent increase in cytoplasmic presence of the RNA-binding protein, HuR [4,40]. Although we observed a trend towards increased spontaneous secretion of IL-6 and TNF-α from human macrophages, the effect was not statistically significant. The discrepancy of our data with those from Chen's *et al.* may depend on the different experimental conditions used, as in that study the effects of Lopinavir were tested in mouse macrophages and THP-1 macrophages [4]. Nonetheless, along

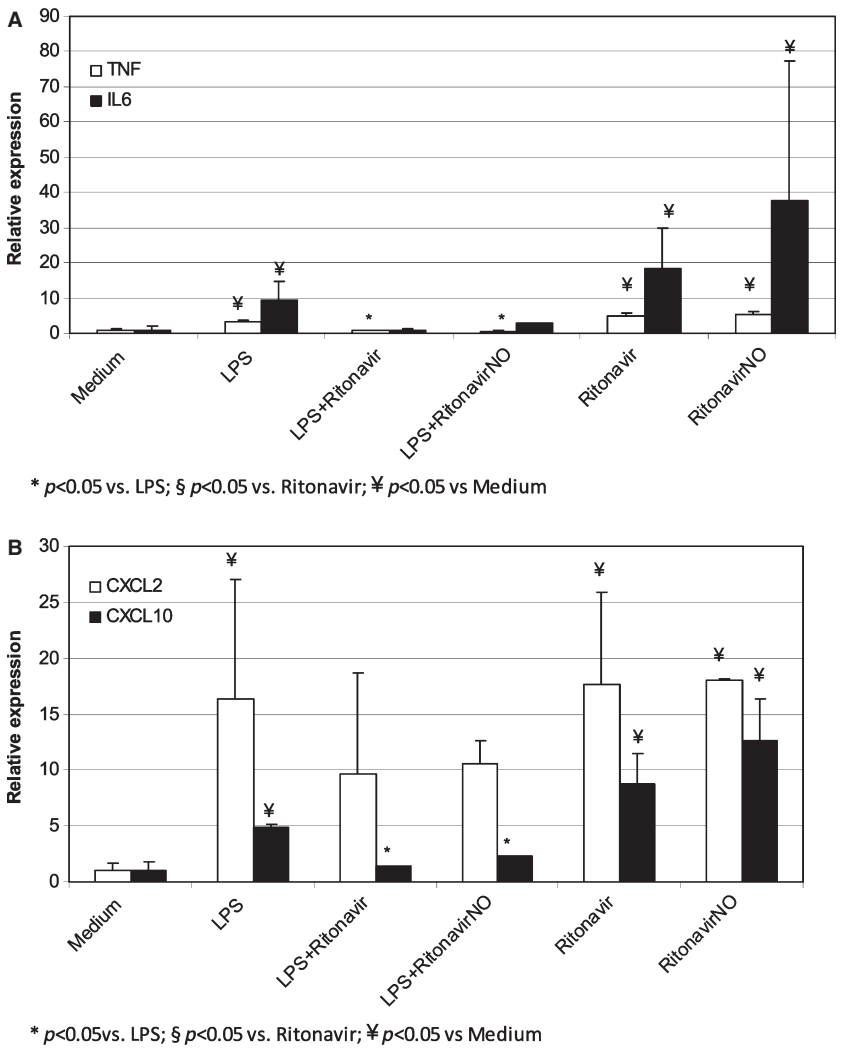

Fig. 7. Comparative effects of Ritonavir and Ritonavir-NO on LPS-stimulated macrophages. Macrophages were obtained from PBMCs of healthy individuals and were stimulated with LPS (10 μg/ml) for 18 hr in the presence of either Ritonavir or Ritonavir-NO for the subsequent extraction of total RNA. Data are from real-time RT-PCR for TNF-α and IL-6 (A) and CXCL2 and CXCL10 (B). Results are from three independent experiments.

with the trend towards increased spontaneous secretion of IL-6 and TNF-α observed in Lopinavir-treated macrophages, the impact of this drug on the function of these cells is highlighted in our study by the up-regulated spontaneous expression of CXCL10 observed in these cells upon co-culture with Lopinavir.

Although not examined in the present study, the effect of PIs treatment on the immune function seems to be related to the potent inhibitory effects of these drugs on proteasome activity [35,41,42], and indeed proteasome blockers by inhibiting transcriptional factors, such as NF-kB, reduce transcription of genes encoding pro-inflammatory cytokines, impair cell-cycle progression and down-regulate T lymphocyte reactivity [43,44].

Our present data, also suggest that, if like Saquinavir-NO, Lopinavir-NO also retains its antiretroviral action, this compound could also successfully replace its parental compound Lopinavir in HAART regimens. In fact, HIV elicits an impressive activation of peripheral lymphocytes of infected individuals [45]. The administration of Lopinavir-NO to these patients could down-regulate the HIV-driven hyperactivation of the immune system. Thus, the recovery of T-cell function may derive not only by the block of viral but also from the direct effect of NO-modified PIs on hyperactivated cells. The immunomodulatory effects of NO-modified PIs could benefit the patients by reducing the susceptibility of CD4+ to immune-mediated damage and apoptosis [46] and down-regulate the production of pro-inflammatory cytokines that may underlie HIV-related disorders, such as dementia [47], non-Hodgkin lymphoma [48] and impaired bone mineral density [49].

In conclusion, our data demonstrate that NO-hybridization of Lopinavir generates a derivative with significantly stronger immunomodulatory effects that are apparently related to an action of the compound on T-cell secretory capacity. The magnitude of the effects observed indicates that Lopinavir-NO deserves additional studies for its possible use in the treatment

of T-cell-mediated autoimmune diseases including, but not limited to autoimmune hepatitis.

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