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Short communication

The new and less toxic protease inhibitor saquinavir–NO maintains anti-HIV-1 properties in vitro indistinguishable from those of the parental compound saquinavir

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ABSTRACT

Although, the antiviral activity, tolerability and convenience of protease inhibitors have improved significantly in recent years, toxicity-associated adverse events including diarrhea, lipid alterations, disturbance of glucose homeostasis and liver enzyme elevations still remain a major concern during treatment of HIV-1 patients. We have recently shown that the covalent attachment of the NO moiety to the HIV-1 protease inhibitor saquinavir (Saq–NO) reduces its toxicity. In this study, we evaluated in vitro the anti-HIV activity of Saq–NO vs. its parental compound Saq. Site directed mutants with the most frequently identified Saq associated resistance mutations and their combinations were generated on proviral AD8-based backbones. Phenotypic assays were conducted using wild type clinical isolates and fully replicating recombinant viruses with Saq and Saq–NO in parallel on purified CD4+ T cells. The following recombinant viruses were generated and tested: L33F, M46I, G48V, I54V, I84V + L90M, M46I + L90M, G48V + L90M, M46I + I54V + L90M, L33F + M46I + L90M. The fold change resistance compared to the wild type viruses was between 1.3 and 7 for all single mutants, between 3.4 and 20 for double mutants and between 16.7 and 28.5 for viruses carrying three mutations for both compounds. The results clearly demonstrate that Saq–NO maintains an anti-HIV-1 profile very similar to that of Saq. The possibility to reduce Saq associated side effects and to increase the concentration of the drug in vivo may allow a higher and possibly more effective dosage of Saq–NO in HIV-1-infected patients and to increase the genetic barrier of this PI thus impairing the selection of resistant clones.

HIV protease inhibitors (PIs) are potent antiretroviral drugs that represent a pivotal component of highly active antiretroviral therapy (HAART) (Fernandez-Montero et al., 2009). Resistance to all available PIs has been observed (Boeri et al., 2003; Johnson et al., 2009; Wensing et al., 2010) and major PI-resistance mutations and minor mutations have been documented (Johnson et al., 2008, 2009). Single mutations have usually only a small effect on resistance (Menzo et al., 2003; Wensing et al., 2010), but during continuous PI therapy, additional mutations emerge in the protease, that lead to high-level of resistance (Johnson et al., 2009; Menzo et al., 2003; Wensing et al., 2010). Since HIV-1 escapes PI treatment by the initial selection of only one or two primary mutations in the protease, an attempt to limit therapy failure has been to 'boost' PI levels in plasma by administration of low doses of Ritonavir to inhibit cytochrome P450-mediated metabolism of PIs (Kempf et al., 1997; Ribera and Curran, 2008). This strategy forces the virus to accumulate multiple protease mutations to become resistant and replicate efficiently in failing patients. Thus, due to the great efficacy of boosted PI-based HAART regimens and the high genetic barrier to resistance, PIs have been considered also in simplification regimens in several trials to reduce the selection of multi-class-resistant variants also in HAART regimens based on novel classes of antiretrovirals (Fernandez-Montero et al., 2009; Wensing et al., 2010).

However, the need for a life long adherence and extensive PIs usage has to face the need for a reduced toxicity of this class of drugs. In fact, even if the antiviral activity, tolerability and

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convenience of PIs have improved significantly in recent years, toxicity-associated adverse events including diarrhea, lipid, and liver enzyme elevations still remain a major concern during treatment of HIV-1 patients with PIs (Boesecke and Cooper, 2008). A common unwanted side effect of PIs relates to their link with the development of insulin resistance and metabolic disturbances that may pose the patients to greater risk for cardiovascular disease. The precise molecular mode of action by which different PIs selectively induce insulin resistance is not precisely known (Hruz, 2008). The inhibition of Akt serine/threonine kinase that transmits the signal triggered by ligation of insulin to its receptor may represent the molecular basis for the toxicity of the PI Saq on glucose and lipid metabolism (Schütt et al., 2004). We have recently shown that the covalent attachment of the nitric oxide (NO) moiety to the HIV protease inhibitor Saq produced a new chemical entity, named saquinavir–NO (Saq–NO), with anticancer properties and reduced toxicity (Maksimovic-Ivanic et al., 2009). Saq–NO does not inhibit Akt with a major advantage over the parental drug in term of toxicity (Maksimovic-Ivanic et al., 2009). Moreover, Saq–NO transiently up-regulated Akt phosphorylation, delivering a protective signal that could be relevant for cell protection and absence of toxicity in vivo (Maksimovic-Ivanic et al., 2009). In previous in vivo studies we have shown that Saq–NO does not provoke lethality even at a dose equivalent to 100% lethal doses of Saq (Maksimovic-Ivanic et al., 2009).

This promising pharmacological profile of Saq–NO led us to carry out the present study where we evaluated head to head the in vitro anti-HIV activity of Saq–NO vs. its parental compound Saq in inhibiting the infectivity of HIV-1.

Saq was purchased from Hoffman-La Roche. Saq–NO was purchased from GaNiAl Immunotherapeutics (Wilmington, DE) and was synthesized as described elsewhere (Maksimovic-Ivanic et al., 2009).

All experiments were performed on freshly purified human CD4+ T cells obtained from healthy blood donors as described (Shen et al., 2008). In brief, peripheral blood mononuclear cells were obtained by Hystopaque–Ficoll gradient centrifugation and then activated with phytohemagglutinin (0.5 mg/ml) and interleukin-2 (100 U/ml) for 3 days. Then, CD4+ T cells were purified by magnetic beads (Miltenyi Biotec, Auburn, CA) and immediately used to evaluate drug activity (Canducci et al., 2010). Experiments were performed in triplicate. PCR site-directed mutagenesis was used to generate viral variants with resistance mutations associated with single and multiple PIs. Mutagenesis was performed on the pNL(AD8) CCR5-tropic proviral backbone, with a proofreading enzyme (pfuUltra II Fusion HS DNA polymerase, Stratagene) and the following primer pairs: L90Mfor (attggaagaaatctgatgactcagattg gctgcactttaaat) and L90Mrev (atttaaagtgcagccaatctgagtcatcagattt cttccaat); I84Vfor (agtaggacctacacctgtcaacgtaattggaagaaatctg) and I84Vrev (cagatttcttccaattacgttgacaggtgtaggtcctacta); G48Vfor ggaa accaaaaatgatagtgggaattggaggttttatca) and G48Vrev (gataaaacct ccaattcccactatcatttttggtttcc); M46Ifor (ccaggaagatggaaaccaaaaat catagggggaattggagg) and M46Irev (cctccaattccccctatgattttggtttc catcttcctgg); I54Vfor (taggggggaattggaggttttgtcaaagtaagacagtatg atc) and I54Vrev (gatcatactgtcttactttgacaaaacctccaattcccccta); L33Ffor (ggagcagatgatacagtattcgaagaaatgaatttgcca) and L33Frev (tggcaaattcatttcttcgaatactgtatcatctgctcc). Ligation mixture was used to transform competent cells (Omnimax cells; Invitrogen) by heat shock. After transformation, bacterial colonies were grown in 5 ml of Luria–Bertani medium, plasmids were extracted by using Promega Miniprep kit and mutagenesis verified by sequencing. Ten wild type subtype B clinical isolates already available in our laboratory were also tested (Paolucci et al., 2003). Drug susceptibility was evaluated as previously described with minor modifications (Brun-Vézinet et al., 1992; Shen et al., 2008). In brief, for each analysis CD4+ T cells were collectively infected with 100 $CCID_{50}$ of each recombinant virus. After 4–5 h of infection, cells were washed with PBS twice and seeded in 96 well plates (100,000 cells/well) in duplicate. Five serial dilutions of either Saq or Saq–NO were then added to the cultures. After 5–6 days supernatant was collected and viral p24 production was quantified as described (Menzo et al., 2003). The inhibitory curves were fitted by non-linear regression (GraphPad Prism), allowing IC_{50} calculation. To evaluate the toxicity of the compound at the tested concentrations, daily cell counts and the metabolic MTT test were also performed in parallel. Statistical analyses were performed by using GraphPad Prism software to evaluate differences in activity and toxicity between Saq and NO-modified Saq.

Recombinant viruses with the following single and multiple PI-associated resistance mutations were generated and tested: L33F, M46I, I54V, G48V, I84V + L90M, M46I + L90M, G48V + L90M, M46I + I54V + L90M, L33F + M46I + L90M. A similar susceptibility was observed with both drugs (Student's t test, $p > 0.05$) (Table 1). The fold change resistance (FCR) compared to the wild type virus was between 1.3 and 7 for all single mutants, between 3.4 and 20 for double mutants and between 16.7 and 28.5 for viruses carrying three mutations for both compounds (Table 1). Toxicity was not observed with the metabolic MTT assay (data not shown), while reduction in cell count was observed with Saq–NO at the highest tested concentrations (χ^2 analysis: $p = 0.47$ at 9.375 μM) (Fig. 1).

The present results demonstrated that the covalent addition of a NO group to Saq has not modified its antiviral activity when tested against a wild type HIV-1 laboratory strain and showed a comparable resistance profile for Saq-resistant clones and an anti-HIV potency with similar IC_{50}.

Since Saq–NO is less toxic than Saq both in vitro and in vivo, larger doses of Saq–NO could be used in the treatment of HIV-1 patients that may allow to increase the genetic barrier of this PI thus impairing the selection of resistant clones (Ribera and Curran, 2008). Moreover, it has been demonstrated that a higher barrier and the absence of development of genotypic or phenotypic resistance protects the other drugs present in the antiretroviral regimen and is directly correlated with a longer viral suppression (Wensing et al., 2010). Saq–No had some cytostatic activity at very high concentrations (about 130 times higher than the IC_{50} of wild type viruses) in vitro. However, since single or double mutants showed a low increase in FCR, Saq–NO may maintain at least in part its effi-

Table 1
Saquinavir and saquinavir–NO IC_{50} (μM) and resistance fold change (FC) respect to wild type virus. Student's t-test analysis was performed to compare the level of susceptibility to Saq and Saq–NO and resulted to be not significant for all viruses tested (95% confidence interval). FC cut-off for our in house assay: low cut-off 2.5 (FC associated with reduction of susceptibility to the drug); high cut-off 13 (FC associated with complete loss of susceptibility) as evaluated by comparison with our previous phenotypic determinations (Menzo et al., 2003; Paolucci et al., 2003). SD = standard deviation.

Mutations	Saquinavir		Saquinavir–NO	
	IC_{50} ± SD	FC	IC_{50} ± SD	FC
Wild type backbone	0.07 ± 0.03		0.09 ± 0.03	
L33F	0.25 ± 0.05	3.5	0.12 ± 0.8	1.3
M46I	0.31 ± 0.10	4.4	0.14 ± 0.8	1.5
G48V	0.49 ± 14	7	0.45 ± 0.10	5
I54V	0.35 ± 0.20	5	0.28 ± 0.12	3.1
I84V	0.14 ± 0.08	2	0.2 ± 0.03	2.2
L90M	0.13 ± 0.07	1.8	0.12 ± 0.03	1.3
M46I + L90M	0.48 ± 0.05	6.8	0.59 ± 0.1	6.5
G48V + L90M	1.4 ± 0.5	20	1.53 ± 0.9	17
I84V + L90M	0.42 ± 0.07	6	0.31 ± 0.12	3.4
L33F + M46I + L90M	1.44 ± 0.33	20.5	1.51 ± 0.08	16.7
M46I + I54V + L90M	2 ± 0.42	28.5	1.86 ± 0.12	20.6
Wild type clinical isolates ($n = 10$)	0.05 ± 0.04		0.06 ± 0.05	