HIV-1 Protease Inhibitors Resistance Profiles in Patients with Virological Failure on LPV/r-based 2nd Line Regimen in Cambodia

Eric Nerrienet*1, Janin Nouhin1, Sophek Ngin1, Olivier Segera2,3, Sreymom Ken1, Kerya Phon1, Chanroeurn Hak2, Vara Ouk2, Vonthanak Saphonn1, Laurent Ferradini1 and Jean-Francois Delfraissy1,4,5

1HIV/Hepatitis Laboratory, Pasteur Institute, Phnom Penh, Cambodia
2ESTHER/Calmette hospital, Phnom Penh, Cambodia
3Clinical Immunology Department, Bicêtre Hospital, Kremlin Bicêtre, France
4National Center for HIV/AIDS and STD (NCHADS), Phnom Penh, Cambodia
5FHI 360 Cambodia, Phnom Penh, Cambodia

*Corresponding author: Eric Nerrienet, Ph.D, Institut Pasteur de Paris, Unité RIR, 25 rue du Dr Roux, 75 045, Paris, France, Tel: 33 6 24 36 83 87; Email: enerrien@pasteur.fr

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Objective: To describe the ARV resistance profiles of patients experiencing virological failure after at least 6 months on LPV/r-based 2nd line regimen in Cambodia.

Design: Retrospective analysis of resistance testing of 89 patients with detectable viral load under LPV/r-based 2nd line regimen.

Methods: Bulk sequencing of HIV-1 protease, reverse transcriptase and integrase PCR products.

Results: Protease gene amplification was successful for 71/89 patients (80%). All were infected by CRF01_AE viruses. Among them, 42 did not present any resistance to PIs. A high level of resistance to PIs was observed for the 29 remaining patients. Twenty-six were resistant to LPVr (8 possibly resistant). Twenty-eight, 21 and 20 were also found resistant to IDV, ATV, and FPVr, respectively. Twenty-six were resistant to NFV (11 possibly) and 22 to SQVr (9 possibly). Finally, 22/29 (75.8%) were resistant to at least 3 PIs. Interestingly, 78.6% (22/29) were found sensitive to DRVr. In this group, a high frequency of resistance to RTIs including ETV was also reported. No resistance to raltegravir (RAL) or elvitegravir (EVG) was observed (n=24). Detailed ARV histories documented for 15 patients revealed past exposition to multiple RTIs and PIs.

Conclusion: Almost 2/3 of patients (60/89) with virological failure on LPV/r-based 2nd line in our study were not in urgent need for treatment change. In contrast, switching treatment was clearly required for 1/3 (29/89) presenting high level of resistance to PIs and RTIs. For those patients, DRV, RAL/EVG, and potentially ETV, could be good candidates for 3rd line ARV regimen if available.

Keywords: Antiretroviral; Second line; LPVr; Protease and integrase inhibitors; Drug resistance; Cambodia

Introduction

HAART treatment programs have proven the feasibility and efficacy of first line antiretroviral (ARV) regimen in resource-poor settings to be similar to those reported in developed countries [1,2]. In Cambodia, 13.6 million inhabitants, the number of HIV-infected patients on reverse transcriptase inhibitors (RTIs)-based first line ARV regimen increased dramatically these last few years, reaching nearby 41,660 by the end of 2010 [3]. Favourable outcomes were reported in Cambodia among adults and children after one to four years of first line ARV regimen associating AZT or D4T, 3TC/FTC, NVP or EFV [4–7]. At the same time, highly active antiretroviral therapy (HAART) duration is increasing and treatment failures become more common leading to a rising number of patients initiated on second line protease inhibitor (PI)-based HAART regimen. As of end of 2010, the National Centre for HIV/AIDS, Dermatology and STD (NCHADS) reported that nearly 1,800 patients already switched to a protease inhibitor (PI)-based 2nd line in Cambodia [8].

Recent WHO recommendations for second line ARV drug regimen include the combination of 2 distinct nucleoside reverse transcriptase inhibitors (NRTI) among didanosine (ddl), abacavir (ABC), tenofovir (TDF) plus lamivudine (3TC), and one ritonavir-boosted protease inhibitor (PI/r) [9].

We recently reported that ritonavir boosted Lopinavir (LPVr)-

based second line ARV regimen in Phnom Penh was associated with a high rate of virological suppression and good immune reconstitution after 24 months of treatment [10].

As expected by the rising number of patients on LPVr-based 2nd line regimen and the extended duration of follow-up on 2nd line regimen, the emergence of 2nd line treatment failures is becoming an important challenge in developing countries with limited virological follow-up and no optional third line regimen [11]. Indeed, mainly because of their price, the number of PI available in these countries remains limited and no integrase inhibitors-based 3rd line ARV regimens are currently available.

The present study aimed to examine the resistance patterns in the protease (PR) gene of HIV-1 in Cambodian patients presenting virological failure on lopinavir boosted ritonavir (LPVr)-based 2nd
line regimen). Such information might help to estimate the need for alternative regimen in Cambodia and to design appropriate alternative protease inhibitor (PI)-based 2nd line or integrase inhibitor (II)-based 3rd line regimen in Cambodia.

Material and Methods

Patients and plasma specimen

By the end of October 2010, 1,888 blood specimens have been referred for ARV resistance testing as routinely performed at Pasteur Institute of Cambodia (IPC). The nature and the duration of the PI-based therapy were collected and recorded along with the samples. Among 130 patients on LPV/r-based second line regimen, 89 experienced virological failure (VL > 250 copies/ml) after at least 6 months on LPV\(_r\)-based 2nd line regimen. Fifty percent of them were patients followed-up by non governmental organizations (NGOs), 30% were referred by the private sector, and 20% by national hospitals. Most of them are regularly monitored for HIV VL and resistance testing at IPC.

HIV RNA Viral load (VL)

Plasma samples were used for HIV RNA viral load (VL) assessment as well as drug resistance genotyping. HIV RNA was extracted from plasma using QIAamp Viral RNA mini kit (Qiagen, Hilden, Germany). The HIV RNA VL was assessed using the G2 Generic HIV-1 viral load ANRS kit (Biocentric, Bandol, France) [12,13]. Using 0.2 ml of plasma, the threshold of the assay was 250 copies/ml.

HIV Drug Resistance Analysis

Bulk sequencing of protease (PR), reverse transcriptase (RT) and integrase (IN) genes were performed according to the complete sequencing procedures and primer sequences previously described [14] in order to detect PI, RTI and II resistance associated mutations (RAMs).

Briefly, nested-PCR amplified fragments were sent to the Macrogen Company (Macrogen Inc., Seoul, Republic of Korea) and chromatograms sent electronically to the Pasteur Institute of Cambodia were verified, analyzed, and interpreted using Ceq2000 (Beckman Coulter) software. Nucleotides and amino acid sequences were verified, analyzed, and interpreted using Ceq2000 (Beckman Coulter) software. Nucleotides and amino acid sequences were compared with the HXB2 reference sequence using Mega 4 [15]. Drug resistance mutations were defined according to the last updated ANRS algorithms [14] taking into account the natural genetic polymorphism of CRF01_AE viruses predominant in South East Asia. HIV subtypes were determined by sequence analysis of amplified fragments using CLUSTAL X 1.81 software [16] and subtype reference sequences set from Los Alamos HIV sequence Database.

Nucleotide accession numbers

Nucleotide sequences of HIV-1 PR, RT, and IN obtained from patients have been submitted to GenBank under the accession numbers JN675743 to JN675813, JN675814 to JN675896, and JN675719 to JN675742, respectively.

Results

By the end of October 2010, ARV resistance profiles of viruses infecting 89 patients (24 females, 65 males) experiencing virological failure (VL>250 cp/ml) after at least 6 months on LPV\(_r\) based 2nd line regimen could be retrospectively analyzed. At the time of blood sampling, the median age of these patients was 37 years [IQR: 26.2-41, range 5-64], the median duration on LPV\(_r\) was 22 months [IQR: 12-41, range 6-144] and the median VL was 4.5 Log\(_{10}\) [IQR: 3.3-5.3, range 2.5-6.8] (Table 1). The most frequent 2nd line regimen prescribed were 3TC-TDF-LPV\(_r\) (n=24, 26.9%), 3TC-DDI-LPV\(_r\) (n=15, 16.8%), ABC-DDI-LPV\(_r\) (n=14, 15.7%), 3TC-ZDV-LPV\(_r\) (n=13, 14.6%), ZDV-DDI-LPV\(_r\) (n=5, 5.6%) and 3TC-d4T-LPV\(_r\) (n=4, 4.5%).

First group of patients with lack of HIV-protease gene amplification (group A)

Three groups of patients could be defined according to their distinct pattern of resistance in the HIV protease genes. The HIV protease gene could not be PCR amplified for 18 (16 males and 2 females) of the 89 patients (20.2%, referred thereafter as group A). Their median duration on LPV\(_r\)-based 2nd line regimen was 18 months [IQR: 13-27; range 6-45] and the median HIV RNA VL was at 2.9Log\(_{10}\) copies/ml [IQR: 2.8-3.4; range 2.6-4.1] (Table 1). The most common LPV\(_r\)-based 2nd line regimen associated TDF or ddi plus 3TC (n=11) or ABC plus ddi (n=3).

In this group, HIV-RT genes amplification was successful for 12 out of 18. In one case no RTI-RAMs were detected while for the 11 others, the most frequent RTI-RAMs were in positions M184 (7, 38.9%), T215 (6, 33.3%), Y181 (6, 33.3%), M41/K101/G190/L210 (4, 22.2%) (Figure 2A). In this group, 50% of patients were resistant to NVP/EFV, 44.4% to 3TC/TFC, and 38.9% to ZDV/d4T (Figure 2B).

HIV-PR PCR products could be sequenced for the 71 remaining patients (79.8%). Phylogenetic analysis indicated that all were infected by CRF01_AE viruses commonly found in Cambodia [17-19]. According to the natural genetic polymorphism of CRF01_AE viruses, all these 71 patients harbored viruses with M36I, H69K and/or L89M substitutions known to confer possible resistance to tipranavir (TPV\(_r\)) [20-22].

Second group of patients with no resistance to PIs (group B)

Another group of 42 of 89 (47.2%), referred thereafter as group B, did not show any resistance to PIs. Their median duration on LPV\(_r\)-based 2nd line regimen was 16 months [IQR: 7.2-30.2; range 6-72] and their median HIV RNA VL was 4.8Log\(_{10}\) copies/ml [IQR: 4.1-5.4; range 2.7-6.8] (Table 1). The most frequent LPV\(_r\)-based 2nd regimen prescribed for this group were TDF or ddi plus 3TC (n=15), ABC plus ddi (n=11) or ZDV plus 3TC or DDI (n=10).

Figure 1: Frequencies of PI-RAMs (1A) and frequencies of resistance to PIs (1B) reported in patients from group B (blue squares), and C (red squares). Asterix indicate the location of the polymorphism mutations (L10, K20, M36, H69 and L89) as reported for CRF01_AE viruses.
Ten out of these 42 CRF01-AE strains did not present any PI-RAMs in exception of the polymorphic ones as described above. Thirty-two patients had viruses with additional PI-RAMs (Figure 1A) which, however, did not confer resistances to PI according to HIV-1 B subtype algorithms [14] (Figure 1B).

When looking at the HIV RT gene of these patients, RTI-RAMs could not be detected for 20 of these 42 patients (47.6%). For the 22 remaining patients, the most frequent RTI-RAMs were located in positions Y181, M184 and G190 (Figure 2A). RT resistance analysis indicated that 20 of 42 (47.6%) were resistant to NVP/EFV, 14 (33.3%) to d4T, 12 (28.6%) to ZDV and 12 to 3TC/FTC (28.6%). Resistance to ABC, TDF and ETV were 23.8%, 11.9% and 7.1%, respectively (Figure 2B).

Third group of patient with high level of resistance to PIs (group C)

A third group of patients (n=29, 32.6%) presented PI-RAMs conferring resistance to at least one PI (thereafter referred as Group C). The median age in this group was 40 years [IQR: 36-42, range 9-55], the median duration on LPV/r-based 2nd line regimen was 35 months [IQR: 24-60, range 9-144] and the median VL was 5Log10 copies/ml [IQR: 4.3-5.4; range: 2.5-6.3] (Table 1). In contrast with what was observed for the other groups A and B, a large majority of males (n=27, 93.1%) was found in this group. Compared with the previous two groups, the median duration on LPV/r was significantly higher (p=0.0001) (Table 1).

The LPV/r-based regimens prescribed in this group were more heterogeneous with some patients receiving ZDV-3TC-TDF-LPV/r (n=3) or double PI-based 2nd line regimen including FPV/r-LPV/r (n=1), NFV-LPV/r (n=1), 3TC-SQV/r-LPV/r (n=1), DDI-TDF-SQV/r-LPV/r (n=1).

Beside HIV-PR gene polymorphism mutations, the most frequent PI-RAMs observed, were found at positions M46 (n=21, 72.4%), I54 (n=17, 58.6%), V82 (n=17, 58.6%), L63 (n=14, 48.3%) and A71 (n=13, 44.8%) (Figure 1A). PI-resistance profile analysis revealed that

![Figure 2](image2.png)

**Figure 2**: Frequencies of RTI-RAMs (2A) and frequencies of RTIs (2B) detected in patients from group A (green squares), group B (Blue squares) and group C (red squares).

![Figure 3](image3.png)

**Figure 3**: ARV resistance patterns of 29 patients experiencing virological failure and developing at least one resistance to PI (group C). Red square = resistant, square red dashed = possibly resistant, white square = sensitive, na= not available.
among the 18 LPV/r-resistant patients was due to the selection of the boosted ritonavir (FPV/r), 26 (89.6%, including 11 only possibly resistant to LPV/r). Such possible resistance to LPV/r was found only possibly resistant to DRV/r while the remaining 22 patients were found truly resistant since they were infected by viruses harboring a high level of resistance to RTIs were detected. The most frequent were in positions M184 (n=22, 75.9%), T215 (n=20, 69%), M41 (n=19, 66.5%), D67 (n=18, 62.1%), V75 (n=17, 58.6%), Y181 (n=14, 48.4%), G190 (n=13, 44.8%) and K101 (n=12, 41.4%) (Figure 2A). Twenty-six patients out of 29 (89.6%) showed resistance to ZDV, 27 (93.1%) to IDV and ATV/r for patient 17. When considering resistances to other PIs (Figure 3, almost all patients) with no resistance to PIs, some patients had viruses with PI-RAMs which, however, did not confer resistances to PI according to the list mentioned above. The remaining 3 patients (patients # 8, # 17, and # 26 in Figure 3) were resistant to ETV (including 6 only possibly resistant). Twenty-one (72.4%) were found resistant to TDF, 22 patients (including 8 possibly) and to DDI for 17 (58.6%, one possibly resistant), to ABC for 22 patients (including 8 possibly) and to DDI for 17 (58.6%, one possibly resistant). Twenty-one (72.4%) were found resistant to TDF, but 17 of them were considered only possibly resistant. Finally, only 13 patients (44.8%) were found resistant to ETV (including 6 only possibly resistant) (Figure 2B, Figure 3).

In order to obtain additional information in this group of patients to design either alternative 2nd line or potentially 3rd line regimen, we performed sequencing analysis of the HIV integrase gene for 24 out of the 29 patients. As already described for CRF01_AE viruses [18,23,24], natural polymorphism mutations in the integrase gene were found in positions D10 (n=23, 95.8%), K14 (n=23, 95.8%), A21 (n=20, 83.3%), V31 (n=22, 91.7%), S39 (n=17, 70.8%), T112 (n=24, 100%), G123(n=24, 100%), T125 (n=24, 100%), R127 (n=24, 100%), G134 (n=24, 100%), I135 (n=23, 95.8%), K136 (n=22, 91.7%), D167 (n=13, 54.2%), V201 (n=24, 100%), N232(n=24, 100%), L234 (n=24, 100%), S283 (n=20, 83.3%) (data not shown). None of these polymorphism mutations are known to be associated with resistance to IIs in HIV-1 B viruses. Four CRF01_AE strains also harbored a V72I HIV-1 RAM which, alone, is not sufficient to induce resistance to raltegravir (RAL). Another HIV-1 strain presented an amino acid substitution in position 92 (E92K) which is not reported to confer resistance to RAL or ETV. Indeed, only the distinct E92Q substitution is known to be associated to RAL and ETV resistance in HIV B viruses. Finally, none of the mutations observed in the HIV integrase gene could be formally associated to resistance to RAL or ETV (Figure 3) in these patients.

Discussion

We report here the antiretroviral drug resistance profiles of patients presenting detectable viral load after at least 6 months on LPV/r-based 2nd line therapy. The first major finding was that no PI resistance was observed for almost two-third of the 89 patients studied outlining the importance of adherence strengthening before envisaging any costly treatment modifications as recently reported [25].

Indeed, the fact that HIV-1 protease PCR amplifications were unsuccessful for 20% of the patients (group A) could be explained by their low level of VL (< 1,000 copies/ml, Table 1) below the sensitivity (1000 copies/ml) of the technique used [14] In the group B (47.2% of the patients) with no resistance to PIs, some patients had viruses with PI-RAMs which, however, did not confer resistances to PI according to HIV-1 B subtype interpretation algorithms. Even we cannot completely exclude the presence of PI-RAMs in viruses infecting patients of group A, these results suggest that wild type or quasi wild type viruses might have emerged in these patients probably because of recent treatment discontinuation or sub-optimal adherence. All together, these 2 groups of patients with no resistance to PIs represented more than 2/3 (67.4%) of patients studied. Interestingly, controlled viral loads performed 6 to 9 months after this first detectable VL revealed that strengthening adherence alone lead to the control of HIV replication for 80% of them (data not shown).

Thus, in Cambodian settings, where few 2nd line alternatives and no 3rd line regimens are available, focusing on adherence strengthening for patients on 2nd line regimen is critical with a control of the viral load after few months following LPV/r initiation (to detect early poor adherence to 2nd line).

On the other hand, a third group (Group C) representing almost one-third (32.6%) of the patients displayed multiple resistances to PIs and clearly required urgent treatment modifications. In depth clinical investigations obtained for some of them revealed they all had experienced multiple PI drugs distinct from LPV/r - more than 10 years ago which might have favored the emergence of multiple PI resistance over time. This is similar to the results of first line treatment failures mostly occurring in ARV-naïve patients in Cambodia [4,26]. In addition, the significantly longer period of follow-up on LPV/r-based
regimen in this group compared to the 2 previous groups (p= 0.001, Table 1) might have allowed the progressive accumulation of additional protease RAMs over time explaining the complexity of the PI resistance patterns observed.

As expected, almost all of the patients of the group C were resistant to LPV<sub>V</sub>, the PI back-bone of 2<sup>nd</sup> line regimen in Cambodia. Patients of group C were also found resistant to many other PIs with the highest number of patient (96.5%) resistant to IDV. Previous exposure to IDV but also the emergence of cross-reacting mutations, such as M46I/L, V82A/F/M/S/T and 184A/V, under the selective pressure of LPV<sub>V</sub> might explain such a high level of resistance to IDV. Indeed, IDV together with SQV<sub>V</sub> and NFV were easily available in the late 90s and often prescribed privately or self administered as initial ARV treatment or after first line failure in Cambodia. Many of these patients presented also resistances to other PIs like ATV<sub>V</sub>, FFV<sub>V</sub>, NFV, SQV<sub>V</sub> or DRV<sub>V</sub> often to 3 or 4 of them at the same time. Similarly to IDV resistance, such a high level of resistance to several PIs might have been cross-selected under extended LPV<sub>V</sub> exposition and also through previous exposure to mostly NFV and/or SQV<sub>V</sub> since other PIs including ATV<sub>V</sub>, FFV<sub>V</sub>, or DRV<sub>V</sub> were and are still not or poorly available in the region.

Only 24.1% of patients from the group C were found resistant to DRV<sub>V</sub> (Figure 1B), including 5/7 patients only possibly resistant. Unfortunately, the ARV histories and the small number of patients followed did not allow us to identify risk factors for development of resistance to DRV<sub>V</sub>. Even some of the patients still sensitive to DRV<sub>V</sub> have already selected one or two DRV-RAMs, the high level of sensitivity (over 75%) observed to DRV<sub>V</sub> made it a good candidate for an alternative ARV regimen for this group of patient.

In order to better figure out the type of potential regimen to be proposed to already PI resistant patients, genotyping analysis of the RT and IN genes gave us more information about still available backbone ARV drugs. ETV was the RTI with the lowest level of resistance even with almost 45% of the patients resistant or possibly resistant, compared to the 25 to 30% recently reported among HIV-1 infected patients failing first and second line treatment in Mali [27]. It should be also added that, among our patients still sensitive to ETV, some already harbored ETV-RAMs which could jeopardize the future virological efficacy of an alternative therapy combining ETV.

On the other hand, no resistance to integrase inhibitors, such as raltegravir and elvitegravir known to be active against HIV-1 resistant to other classes of antiretroviral drugs [28-33], were detected.

For these patients under confirmed 2<sup>nd</sup> line treatment failure (group C) an alternative ARV regimen should be considered case per case considering each treatment history. However, if we consider the possibility of a true 3<sup>rd</sup> line regimen at least, two sensitive ARV drugs should be considered including a new class of ARV, like the integrase inhibitors. As previously reported [29], the combination of DRV/ RAL and potentially ETV could be considered as a 3<sup>rd</sup> line option. Interestingly, 2 patients (#28 and #29, Figure 3) who started ETV/DRV/ RAL therapy had an undetectable VL after 3 to 4 months later.

For such patients who currently need to be switched from their 2<sup>nd</sup> line regimen, the poor availability of alternative ARV class drugs such as ETV/DRV/RAL and its extremely high cost (2000 USD/month) in Cambodia remain dramatic barriers for their survival.

Most of the 29 patients studied here have been previously exposed to several RTI and PI probably at the beginning of the epidemic in Cambodia when ARVs were not yet officially available. It is possible that less people will truly fail to the current 2<sup>nd</sup> line regimen since the vast majority of patients currently initiated on such regimen through the National Program in Cambodia were ARV-naïve before starting first line regimen and probably were never exposed to PIs. This will have to be confirmed by further prospective long term evaluation of 2<sup>nd</sup> line efficacy in Cambodia.

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Conflicts of interest

EN, JN, SN, OS, KP, CH, VO, VS, LF and JFD declare that they have no competing interests.

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Author’s contribution: EN, JN and JFD conceived of the study design. All authors generated the data. JN, SN, SK and KP performed the analyses and interpretation of data. EN, JN and LF wrote the manuscript.

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